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*Basic Bacteriology*

AND ITS

BIOLOGICAL AND CHEMICAL BACKGROUND





# BASIC BACTERIOLOGY

## *Its Biological and Chemical Background*

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BALTIMORE • 1953

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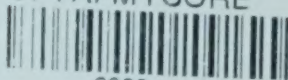
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THE WILLIAMS & WILKINS COMPANY  
BY THE  
WAVERLY PRESS, INC.  
BALTIMORE 2, Md., U.S.A.



## Preface

This book is intended to present the nature of the cytological, morphological, taxonomic, physiological, and biochemical problems which confront the bacteriologist. From this survey it should be evident that the worker in bacteriology requires knowledge of general biological, chemical, and physical principles if he is to be competent in his own area of specialization.

Departments of bacteriology generally require that their major students receive some preparation in these basic sciences. Yet, the courses in which one acquires the necessary background for an approach to bacteriology do not treat the principles presented from the point of view of the interests and needs of the bacteriologist. Thus, we feel that a need exists for an oriented treatment of the information acquired in general physics, chemistry, and biology courses. This is the justification for our treatment of fundamental physical, chemical and biological problems in a bacteriology text.

Inasmuch as a large number of textbooks are available at the elementary level and numerous monographs and reviews have been written at the most advanced level, it has been our desire to bridge the gap between these two sources of information. Therefore, our treatment has been aimed at producing a work of intermediate complexity. It is assumed that the reader has mastered the basic vocabulary of biology and chemistry and has already had some experience in the laboratory with the techniques and materials of bacteriology.

Out of our experience with students, particularly those intending to do graduate work in bacteriology and in biochemistry using bacteria as tools, we have drawn the conclusion that the available textbooks tend to emphasize the same subject matter. As a result, certain lacunae that should be filled exist in the source books used by students. In an attempt to meet this need we have introduced subject matter new to textbooks of bacteriology and treated certain traditional matters with a different emphasis than exists in the popular texts. In the light of this objective we have no desire to duplicate satisfactory texts but rather hope to supplement and round out the treatments already in existence.

An ever-present deficiency in education is the failure to stress the necessity for some grasp of the overall significance of general ideas in order that fundamental principles may be employed in achieving an understanding of diverse topics. Consequently, in the earlier chapters a certain amount of



"elementary" but basic material is included in order to provide a fully integrated picture of the subject under discussion. To help the reader integrate ideas we have included simple things at various points and attempted to develop from these elementary concepts the more complex ideas present in the same or later chapters. We have attempted to explain bacteriological phenomena rather than to merely state their occurrence.

Few compendia of data are presented, and we hope to have succeeded in emphasizing ideas and principles rather than factual knowledge. Specific facts are introduced only as they make a contribution to the development of knowledge of principles and as they may be employed to illustrate established principles. In general the use of graphic illustrations has been favored rather than tabulated data.

It is also our hope to communicate some insight into the nature of the general methodology of science. For this reason we have injected a measure of scientific philosophy into various portions of the work and have treated critically some of the problems, methods, and data of bacteriology. In the latter cases we have chosen situations which seemed particularly suitable for the purpose.

We believe that the science of bacteriology has matured to the extent that a textbook need not be cluttered with references. In other sciences it has been generally appreciated that certain principles and data exist which are accepted by all serious investigators and that it serves no essential purpose in these fields to refer the student to the original literature. We have adopted this attitude for the areas of bacteriology considered. On the other hand, we must admit that the situation in bacteriology is more fluid than in some of the older sciences; therefore, we have not entirely abandoned reference to the original literature. In order to avoid interruptions of the flow of thought occasioned by the inclusion of any large number of references within the body of the textual material, we have listed essential references and review articles at the end of each chapter as a guide for further reading. Papers outside the bacteriological journals which may be useful to the bacteriologist have been included.

We wish to take this opportunity of expressing our thanks to Doctors Roger M. Herriott, Winston H. Price, and Sol H. Goodgal for their constructive criticism of the chapter on bacterial genetics. In particular we wish to thank Doctor Jack J. R. Campbell for the great assistance rendered us in the preparation of the chapter on metabolism and for his suggestions on other portions of the book.

For the use of copyrighted materials we wish to thank the following publications and publishers: Academic Press Inc., N. Y. (Figs. 6, 35, 46, 48, 49); Rockefeller Institute for Medical Research (Figs. 21, 33, 61); Reinhold Publishing Corp., N. Y. (Fig. 23); The Blakiston Co., Phila-



delphia (Fig. 29); The Clarendon Press, Oxford (Figs. 32, 39, 40); Hermann & C<sup>ie</sup>, Paris (Figs. 38, 47, 79); Cambridge University Press (Figs. 54, 55, 56); American Chemical Society (Fig. 82); Journal of Biological Chemistry (Fig. 80); Elsevier Publishing Co., Amsterdam (Fig. 81); Edward Arnold & Co., London (Figs. 41, 42); E. & S. Livingstone, Ltd., Edinburgh (Fig. 65); Journal of Cellular and Comparative Physiology (Figs. 58, 59, 60, 62, 78); Biological Bulletin (Fig. 52); Journal of Pathology and Bacteriology (Fig. 57); Journal of Hygiene (Fig. 97).

# Foreword

The teacher of advanced courses in bacteriology usually is faced with one or both of two unfortunate facts: the students do not know enough about the biological principles of bacteriology and about bacteria as such; and the students are not familiar with the necessary essentials of physics and chemistry, or at least some of the particular aspects of these subjects that apply especially to bacteriology. Although books dealing with the most advanced and specialized phases of bacteriology must, in order to keep within proper bounds, assume a proper preparation on the part of the student, this is rarely the case. The authors of this volume have attempted the difficult task of writing a book that will fill the void between those texts that deal in a very elementary way with the essentials of bacteriology, and those, on the other hand, that treat the advanced aspects of the subject.

Although a greater integration in the teaching of the various sciences is doubtless possible and desirable, it is too much to expect that the sciences of physics and chemistry, which have so many and so important applications in the other sciences and in everyday life, can teach all of the specialized phases that are of importance to students in the various branches of biology. It is therefore essential for the student in biology to have some familiarity with chemical and physical principles and phenomena that are not emphasized in the conventional courses that are taught to the undergraduate college student. Teachers of bacteriology are not alone in feeling a need for supplementary reference volumes in order to fill in the deficiencies in the training of their students. It is exactly this need on the part of teachers of students of medicine, physiology, and biochemistry that has led in the past to the writing of books dealing with such subjects as the physical chemistry of proteins and colloidal chemistry in physiology and medicine, and more recently to such an invaluable classic as *Topics in Physical Chemistry* by William Maunsfield Clark.

The present volume, *Basic Bacteriology and its Biological and Chemical Background*, the result of the combined efforts of a bacteriologist and a biochemist who have shown brilliance and versatility in their own research, should be of great value to both students and teachers of bacteriology.

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## CHAPTER I

# The Scope of Bacteriology

Bacteriology is, of course, the study of particular kinds of microorganisms, the bacteria. It is the science in which is summarized mankind's scientific knowledge of particular kinds of living organisms. As such it is but one aspect of man's curiosity about the universe of living organisms and only one subdivision of the science of biology. Scientific knowledge is the accumulation of factual data about the universe, gained and verified by observation and experiment, and reduced by means of ordered and logical thinking to laws of nature descriptive of the relations of things and events. Bacteriology as a pure science is concerned with the study of the morphology, physiology, and taxonomy of particular microorganisms and their occurrence, variation, heredity, and evolution. It includes the integration of knowledge of physics, chemistry, and general biology with a particular aspect of biology, and on occasion is the vehicle by means of which some general biochemical or biological relation is discovered or verified.

It is in the nature of science that in its infancy it can only be concerned with objects obvious to man in his natural environment. It is only as instrumentation develops and extends man's senses beyond the immediately perceived; that science can concern itself with the less apparent objects and events of nature and the more subtle relations existent between things. Inevitably, this evolution has meant that bacteriology developed as a science long after scientific study of other organisms had begun. It has also meant that the original students of bacteriological phenomena had received their training in other sciences. This breadth of experience reflected to the immense benefit of bacteriology, since investigators could make unusually rapid progress in extending knowledge of general applicability from other fields to bacteriology. It has also resulted in the fact that bacteriology never has had a tradition of conservatism in the extension, practical application, and adaptation of the technics and theoretical knowledge of other sciences. In view of this historical background, and because bacteria as sources of material for study present certain unique advantages, particularly in both their rapid growth rate and the technical feasibility of amassing large numbers of organisms economically, bacteria have been and are becoming increasingly important as objects of study in many different sciences.

But if the nature of the historical development of bacteriology has had advantages sometimes denied in degree to other sciences, it has also had



its disadvantages. The chief of these has been a relative lack of creative interest in bacteria as such. There has not been in existence a great pool of scientific personnel studying bacteria out of a love for the bacteria. Thus, as the applications of bacteriological knowledge became evident, and they became evident early in the history of the science, major emphasis shifted to applied bacteriology. A person was trained as, and felt himself to be, not a bacteriologist so much as a medical bacteriologist, dairy bacteriologist, soil bacteriologist, etc.

The study of pure bacteriology is motivated by the desire to understand the nature of bacteria and their place in the scheme of the universe. Practical bacteriology seeks to control the harmful activities of bacteria and direct their useful activities. The line between applied and pure bacteriology is tenuous, and no universal agreement can be expected as to where it should be drawn. Nor would any great purpose be served by any strict definition of the difference between the two phases of the science. But it is useful to recognize that progress in any science, including bacteriology, and its practical utility for society, is most rapid when there is some balance of effort expended on practical phases and the esoteric or academic studies. Each category of investigation feeds the other. Applied science often helps in the discovery or more precise definition of phenomena. It makes possible the creation of the material means and leisure time that society can invest in the pursuit of pure science for its intellectual satisfactions. Pure science provides the rational basis for practice and suggests new applications. When technology becomes more than merely empirical in methods and outlook, it is because pure science has provided a firm foundation of factual data and theoretical concepts which practicing engineers, technologists, and physicians have mastered.

The science of biology can be divided into sub-sciences by a number of means. The generic system which separates bacteriology as a discipline is the consequence of a logical recognition of one of the historical trends in the development of biology. It is inherent in the nature of biological studies that as the body of biological knowledge expands, individual students of life will tend to concentrate their studies upon particular and phylogenetically related organisms.

The recognition of living forms invisible to the unaided eye was a dramatic experience in the intellectual history of mankind. So it is no wonder that a word, *microbe*, was invented (1878) to describe these apparently related organisms, and there eventually developed the concept of microbiology as a separate science. But microbes form a large group of very diverse organisms, so that their study is actually the fundamental task of virologists, bacteriologists, mycologists, algologists, protozoologists, hel-

minthologists, etc. The diversity of forms properly called microbes has meant that in recent times few persons, if any, have mastered the essential components of all the sciences that can be catalogued under the heading of microbiology. The technics used to study bacteria have had wide application in the study of other microbes, so that there is possibly more in common between the technics of the microbiological sciences than between the phylogeny of the organisms studied. In any case, the lexicographer, if not always those calling themselves microbiologists, has recognized the limitations so that a popular dictionary notes that the term microbiology as generally employed is synonymous with bacteriology. In this sense the use of the term bacteriology is to be preferred.

If a generic concept is to be used in defining the sub-branches of biology there is no compelling logic in considering microbiology as a distinct science. Certainly there is no reason more compelling than those which have prevented anyone from volunteering to father the sibling science, the monstrosity which would be labeled *macrobiology*. The use of common technics, the discovery of general laws, provided the stimulus for thinking in terms of a unified science and the invention of the concept of a science of microbiology. But this term, generic in its implications, violates the logic of a generic system of separation of the biological sciences. Yet the boundaries between organisms must be crossed and identified. For this purpose it would seem better to think in terms of sciences of systematics such as comparative cytology, comparative physiology, and comparative biochemistry. This latter cataloging is also more truly descriptive of the actual situation of the social organization of the sciences. It permits the use of restricted labels with more scientific meaning than the term microbiology. And also important, because science is an agency in the hands of men, its use would help preserve scientific modesty.

The efforts of bacteriologists may be conveniently listed as occurring in five areas of activity:

1. *General or pure bacteriology*. This would include the studies devoted to understanding the fundamental nature of bacteria and their relations to one another and to other organisms.

2. *Soil bacteriology*. Studies in this field have contributed particularly toward understanding of the problem of the fertility of soil and the natural mechanisms for the cyclic biological utilization and deposition of elements in soil and large bodies of water.

3. *Medical bacteriology*.

- A. Animal pathology

- human

- veterinary

- B. Plant pathology
- C. Public health science
- D. Sanitary engineering
- 4. *Food technology.*
  - A. Dairy
    - manufacture
    - control
  - B. Control
    - canning industry
    - other preservation industries
      - refrigeration
      - hypertonic solutions
        - sugar
        - salts
        - chemical
          - acid
          - spices
  - C. Fermented food products: alcoholic beverages, vinegar, pickles, sauerkraut, silage.
  - D. Microorganisms as sources of nutrients: protein, fat, and vitamins.
- 5. *Industrial chemical or fermentation technology.*
  - A. Production of chemicals: glycerol, acetic acid, citric acid, industrial solvents (acetone, ethyl, butyl, and isopropyl alcohols), enzymes, antibiotics.
  - B. Microbiological treatment of industrial products: tanning of leather, retting of hemp, curing of tobacco, coagulation of latex, disposal of industrial wastes.

Bacteria have been considered or employed in many areas of science. These efforts may be conveniently listed and illustrated as follows:

1. *General problems of biology.* The development of bacteriology made possible the definitive overthrow of the theory of spontaneous generation, an event of major philosophic import in the history of biology. Discussions of the origin, nature, and evolutionary development of protoplasm inevitably draw upon bacteriological knowledge.

2. *Physiology.* Bacteria are being increasingly considered for use as a tool for the solution of fundamental problems in genetics, cellular physiology, and comparative biochemistry. Progress in the study of intermediary metabolism has been particularly advanced by the study of metabolic systems of bacteria.

3. *Chemistry.* Besides helping pose many questions with which chemistry must concern itself, bacteria have had important uses in analytical chemistry. Employing bacteria, many sensitive and specific methods have been



developed for the analysis of carbohydrates, amino acids, and vitamins. It is probable that bacteria will find uses in analyses for trace elements. Bacteria have also been used in the separation of optical isomers or their synthesis.

4. *Geology*. Bacteria have been used for the detection of petroleum deposits. In addition, the possibility that bacteria are involved in the formation of petroleum is being actively studied. Bacteria have been considered, so far unsuccessfully, for use in increasing yields from petroleum-bearing sands and strata. Apart from petroleum geology, the role of bacteria in the formation of certain deposits such as chalk and iron oxide and in the erosion of rocks are other subjects of interest to the geologist.

5. *Pest control*. Bacteria, pathogenic for insects and rodents, have been suggested and in a few cases successfully used, for controlling the depredations of these pests.

There is in addition to the listed endeavors of bacteriology, an occasional and unexpected occurrence which excites unusual public attention, the explanation for which may be bacteriological. The most dramatic instances of this kind were the epidemics of "bloody" bread and cereal foods that caused great consternation in the peasant communities of Mediterranean countries a hundred years ago and more. While originally attributed to visitations of the devil in payment for the sins of the communities, bacteriologists determined the "bloody" spots to be masses of growth of the red-pigmented bacterial species, *Serratia marcescens*, growing in response to unusually humid atmospheric conditions.

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## CHAPTER II

# The Occurrence and Taxonomy of Bacteria

### THE OCCURRENCE OF BACTERIA

Wherever they have been sought bacteria have been found on the surface of the earth, both land and water surfaces. If any generalization can be made in regard to the geographical distribution of bacteria, it is that the types found are not peculiar to particular locations. Thus the kinds of bacteria found in polar zones are similar to those encountered in more temperate regions. The fundamental homogeneity of types of bacteria on the surface of the earth is perhaps not surprising when it is recalled that three powerful natural agencies are available for the spread of bacteria, namely: wind, ocean currents, and migratory animals. Over the eons of time that bacterial species have been in existence, there would seem to have been ample opportunity for these agents to contaminate the remotest areas with organisms from originally limited habitats. Actually, it is only a matter of conjecture that particular bacterial types have evolved in restricted territories. The point is that if this hypothesis were correct, natural forces are operating against the probability of organisms remaining confined to a given locality for a long time. There is, then, no logical difficulty in explaining the ubiquitous character of the distribution of bacteria.

Whether an organism can actually maintain itself in a new locality to which it has been transported is dependent upon its capacity for adaptation to the physical and chemical characteristics of the new environment and to the competition with native species of living organisms. Though the kinds of bacteria distributed throughout the earth's surface are related, there are differences in the exact species of bacteria found, and more particularly in the relative and absolute numbers of organisms. In hot springs, thermophilic species may be present which are absent in fresh water lakes, but the thermophilic species found in hot springs a continent apart are similar. The depths of the oceans and other large bodies of water that remain at a fairly low temperature throughout the year have a bacterial flora more abundant in psychrophilic organisms than do smaller bodies of water fluctuating in temperature with the seasons.

In the case of parasitic species it is self-evident that their distribution will be coincident with the normal habitat of the host species. Whether the parasites will be found outside of the bodies of the host species will depend upon how well they are able to survive a separate existence. There exists a



spectrum of bacterial species from those completely dependent upon the host, with only a transient existence outside the host, to those species like *Clostridium sporogenes* for which it is difficult to know whether they are fundamentally soil or intestinal bacilli.

Bacteria not only have a horizontal distribution with respect to the earth's surface but also a vertical distribution. They have been isolated from the atmosphere at altitudes of 12,000 meters. At such heights the number of organisms is small and limited to the types that can resist the cold and ultraviolet irradiation. This latter factor would seem to be a formidable barrier to the long-term persistence of individual bacteria in the atmosphere. Studies have shown that above 500 meters there is a rapid falling off in the number of bacteria.

The kinds of bacteria found in air, apart from confined spaces associated with animal habitations, are marine and soil types, with spore-formers and chromogenic cocci predominating in the air above land masses. The air above the ocean and other large bodies of water has far fewer organisms than does air above land surfaces. The number of bacteria and the extent of their aerial distribution would seem to be entirely dependent upon the vagaries of meteorological conditions, especially since bacteria and their spores have no capacity to grow and multiply in the atmosphere, and unlike many other organisms have no special structures or means for rendering them air-borne.

In soil, bacteria are most abundant in the first few inches of topsoil, the number and kinds varying widely with the nature of the plants present, the chemistry and physical structure of the soil, and the degree of contamination with the detritis of dead plants and animals and excreta of animals. Ground waters, as well as surface waters, contain bacteria. An important factor in the bacterial content of ground water is the nature of the subsoil the waters are traversing. For example, sandstone would tend to reduce the number of bacteria by a filtering action, while limestones with channels dissolved out by the action of water would permit the free passage of bacteria.

Bacteria have also been found at great depths beneath the land surface. These have usually been revealed in deep mines or by study of materials obtained in the process of drilling oil wells and artesian wells. The types of bacteria found in these cases have been primarily nitrate- and sulfate-reducing organisms. In the interpretation of data obtained from these studies, a formidable problem is presented by the possibility of penetration of bacteria from higher strata. However, a reasonable case has been made for the natural occurrence of bacteria at depths as great as 2000 meters. An interesting problem is presented by the aerobic nature of a part of the flora present at this great depth, which is presumably lacking in free atmospheric oxygen.

To explain this apparent anomaly it has been postulated, but not proved, that free oxygen is available from the action of rays from radium and mesothorium on water.

The depths of the oceans contain bacteria, the nature and number of which at particular places is in part dependent upon the proximity of drainage from land masses. The species presumably indigenous to ocean water are predominant or exclusively present where this influence is meager or absent. Studies of cores from beneath the sea floor at great depths have revealed the presence of bacteria. But studies of this sort have not been large in number and are not truly representative of the tremendous extent of the sea bottom. It is difficult, therefore, to make a realistic guess as to how deep beneath the ocean floor the biosphere extends and includes bacteria.

In the sea there is a vertical stratification in the distribution of bacteria. Several studies have shown the highest counts to occur between 25 and 50 meters of depth, with a rapid drop in count to a minimum at about 200 meters. The number then rises abruptly at the interface between the sea and the bottom mud. The autochthonous species of the sea are predominantly gram-negative rods and motile. Cocci constitute a minor portion of the marine flora.

Sedimentation is a natural, purifying mechanism which influences the vertical distribution of bacteria in both air and water. In these relatively non-viscous fluids the tendency would be for bacteria to settle or sediment at a rate that can be calculated from Stokes' law (see Chapter 3). The presence of any solids or particulate matter, generally found to possess an affinity for bacteria, would aid the sedimentation of bacteria.

The study of the geographic occurrence of bacteria is one aspect of bacterial ecology, a poorly cultivated aspect of bacteriology. The study of bacterial ecology is fraught with difficulty but is one in which the present-day scarcity of data promises unexploited opportunities to the imaginative investigator. The natural bacterial inhabitants of an environment can be sought for directly by the combined use of staining technics and the microscope. But such studies are entirely dependent for information on the differing morphological characters of bacteria and do not, therefore, lead to any accurate or detailed description of the normal flora. Consequently, students of bacterial ecology have expended the most effort and placed the most trust on culture technics. In these studies the *elective* or *enrichment culture* technic introduced by Beijerinck has been most productive of reliable data.

The principle of the enrichment culture is the employment of predetermined conditions which will permit the favored or exclusive development of particular kinds of bacteria. The intelligent application of this principle



requires sound knowledge of the differing physiological capabilities of organisms. Enrichment cultures can contribute not only to knowledge of the occurrence and competition of organisms but also to an understanding of basic cellular processes.

When an organism is found in a particular sample by some culture technique there can be no question as to its presence. One need only eliminate the possibility of contamination before concluding that the organisms found are naturally present in the sample. However, a negative finding may have little scientific value, for the failure to observe the growth of a particular type of organism may only mean that conditions for its isolation have not been met. Negative results with enrichment techniques might permit a little more confidence in the value of a negative result but should not lead to the drawing of definitive conclusions based solely on these results. The value of a negative result in an enrichment culture is in direct proportion to our knowledge of the habits and physiology of the organism being sought. Since our knowledge of any organism remains incomplete, the failure to detect growth cannot be taken as conclusive proof of the non-existence of the species sought for in the sample. Nor can any dogmatic faith be placed in logical deduction to predict the nature of the organisms present from a knowledge of the nature of the environment. Experience has shown that, while prediction of the kinds of organisms present may be accurate, prediction of the absence of particular organisms is risky. Two illustrations may suffice to emphasize the points made.

- 1) Many top soils contain a variety of obligate anaerobes in large quantities in spite of the ready presence of air. Certainly from our knowledge of the characteristics of these organisms the observation is unexpected. The common explanation for this finding is that the presence of organisms utilizing oxygen creates local micro-areas of anaerobiosis in which the anaerobes can grow.

- 2) The depths of the sea and sea floor are in most areas at a fairly constant low temperature. It is logical to expect that strictly thermophilic organisms will not exist in these situations. Actually the isolation of thermophiles has been reported even from a 70-inch core dug from the sea bottom 30 miles offshore at a depth of 1310 meters where the temperature is lower than 10°C. No satisfactory explanation for these data has been presented.

To accurately map the bacterial flora of the earth is a task that still remains. There have been no concerted long-term efforts in this regard. Indeed, the value of any attempt to search the literature in order to compile a list of claims made for the isolation of a particular species would be insufficient and in many cases useless. The lack of agreement as to the proper characterizations of species and a varied nomenclature work against the usefulness of such a compilation. The more difficult course, but probably



the most productive in the long run, will be for specialists to cooperate on an international scale in an effort to expand the sources of their cultures. In addition, agreement on problems of nomenclature and a greater interest by all bacteriologists in taxonomic problems will find reflection in an expanding knowledge of bacterial ecology.

### THE SCIENCE OF TAXONOMY

Taxonomy is usually defined as the science which treats of the arrangement and classification of plants and animals according to their natural relationships. Implicit in this definition is the need for knowledge of the natures of the organisms to be classified and for the creation of a system of naming organisms which serves to express the objects and conclusions of taxonomy with an economy of words.

A clear and profound understanding of taxonomy may be approached by a consideration of the three purposes attributed to taxonomy. How well these purposes are satisfied is a measure of the scientific success of a classification.

1) *Definition.* Taxonomic knowledge aims at obtaining a definition of organisms and of their past and present genetic relationships. Taxonomy, which is often charged by the uninitiated as being too theoretical a science, is actually a very pragmatic science since it deals with what *is* rather than with what *can be* in nature. Obviously, any ultimate fulfillment of the purpose of definition would require a complete and absolute knowledge of the biology of organisms. This kind of knowledge is unobtainable at any stage in the development of any science. No scientist is egoist enough to argue that human experience has arrived at the stage where further progress in any science is impossible because we have learned all there is to be learned. If such a state of belief were ever to be accepted there would be no need for scientists but only for technicians and encyclopediasts. Because of the incompleteness of knowledge taxonomy is, and should be, constantly in a dynamic state. To tell what a thing is with precision, to define the properties and relationships of organisms, requires a truly impressive breadth of experience on the part of taxonomists. Every bit of information helps reveal the true nature of organisms. Each datum must ultimately be placed and weighed for its relative value in defining organisms and their relationships. Whether there is conscious realization of the fact or not, all persons scientifically investigating organisms are making a contribution to the data of taxonomy. In a very real sense the best taxonomy is a synthesis of all knowledge of biology. For these reasons the person who complains about the lack of stability of taxonomic science or who would relegate taxonomic interests and researches to the dust bin reveals a rather simple-minded attitude.

An ideal definition whether it be one in taxonomy or elsewhere must have certain characteristics. These are: (a) a definition should be restrictive and exclusive. It should only define the thing to be defined. (b) A definition must not include the thing being defined as a part of the definition. In other words, it should not be circular. (c) A definition should be stated in positive terms. It should specify what is, rather than what is not. (d) Language chosen to frame a definition should not be obscure or ambiguous. A definition in science would exploit scientific vocabulary to the fullest extent since the invention of scientific terms involves a conscious and stern effort at precision in meaning.

2) *Diagnosis*. Taxonomy provides for the identification of organisms. This purpose is *determinative* and as such is a very practical aspect of taxonomy. In this role taxonomy is an instrument serving the interests and needs of other biological sciences.

In order to tell things apart taxonomy must invent the means of comparing organisms and dividing them into groups. Since organisms are genetically related in spite of their differences they have a relationship one to another that should be indicated by some serial method of division, the distance between divisions being indicative of the relative relationship. Ideally, the relation of these divisions would express the degree of past as well as present genetic relationship. Taxonomy should attempt in practice to meet the following rules of logical division: (a) Any given division should be exhaustive. It should not exclude any related thing. (b) Division must also be exclusive. In any given division unrelated objects should not be included. (c) There should be consistent application of guiding principles for the establishment of given levels of division. Thus ideally, principles for the establishment of families would be the same irrespective of the orders of organisms being investigated. (d) The criteria for divisions should be chosen so that they will indicate to the novice, as well as the expert, the traits of most significance in distinguishing organisms, some systematic knowledge of the relationships of organisms, and an indication of the hypotheses about the nature of the things which have led to the particular mode of division. In summary, the divisions must not be a mere serving of convenience; they must have a logical content.

3) *Nomenclature*. Taxonomy provides a systematized catalogue of suitable names. Naming an organism is an act of simplification in communication. Basing the nomenclature upon some universally agreed upon principles provides the means of communicating with an economy of words something about the nature of organisms. Apart from the obvious need of a scientist for knowing accurately with which organisms his colleagues are working, agreement as to the solutions of problems of nomenclature is necessary if the knowledge of one generation is to be transmitted without loss to suc-



ceeding generations. Certainly this is a utilitarian aspect of nomenclature that justifies a liberal attitude by the beginning student toward the memorizing and exact use of the scientific names of organisms. For the experienced and productive scientist concern with problems of nomenclature will have its practical reward in preventing the early and unwarranted assignment of his work to limbo upon his demise.

### THE TAXONOMY OF BACTERIA

The Botanical Code developed by International Congresses of Botanists has been generally accepted by bacteriologists for use in systems of nomenclature in bacteriology. The Botanical Code has been preferred to the Zoological Code since with minor modification it is suitable for application to bacteria which as a group are regarded as being more closely related to plants than animals. The codes of nomenclature are formal statements of principles, rules, and recommendations governing the invention of scientific names and their priorities. Each individual organism is assigned a Latinized binomial name. The specific name spelled with small letters locates the species, and the generic name preceding the specific name is written with a capital first letter and locates the organism within the system of classification. Every individual organism belongs to a species, each species to a genus, each genus to a family, each family to an order, each order to a class, and each class to a division. Depending upon the complexities of particular groups of organisms, available knowledge, and the tastes of taxonomists, these major groups may or may not be subdivided. The grouping established by the Botanical Code is as follows:

#### *Subdivision*

#### *Name*

Kingdom.....	} Names are taken from one of the chief characteristics of the group and expressed by words of Greek or Latin origin.
Division.....	
Subdivision.....	
Class.....	
Subclass.....	
Order.....	Name of one of principal families and ending <i>ales</i> .
Suborder.....	Name of one of principal families and ending <i>ineae</i> .
Family.....	Name of one of genera and ending <i>aceae</i> .
Subfamily.....	Name of one of genera and ending <i>oideae</i> .
Tribe.....	Name of principal genus and ending <i>eae</i> .
Subtribe.....	Name of principal genus and ending <i>inae</i> .
Genus	
Subgenus	
Species	
Subspecies	
or variety	



Though there is tacit agreement to use the International Rules of Botanical Nomenclature in building systems of classification of bacteria there is no universal agreement among bacteriologists to use a particular one of the numerous classifications which have been proposed. The most frequently used classification is probably Bergey's Manual of Determinative Bacteriology, which for a short time was sponsored by the Society of American Bacteriologists and is now governed by a self-perpetuating board of trustees. In spite of its many admitted deficiencies Bergey's Manual deserves this popularity because of its scope, ready availability, and the continuous effort expended at publication of improved editions.

#### WHAT ARE BACTERIA?

In general, bacteria have been classified as plants, the reasons for this decision resting on four chief considerations.

1) As a group, bacteria have plant-like synthetic capacities. Starting with the simplest substrates such as carbon dioxide, ammonia or other inorganic nitrogen compounds, and small amounts of a miscellaneous mixture of inorganic salts, the complex molecules of protoplasm can be synthesized by many species. Even in the case of bacteria requiring more complex raw materials a smaller variety of essential organic compounds is necessary than is needed by animal species.

2) Foods must penetrate the bacterial cell in soluble form in order to be assimilated. When solid foods are utilized by bacteria it is by means of an enzyme mechanism that hydrolyzes or solubilizes the food outside the bacterial cell. The only known exception may be the species of *Thiobacillus* which utilize elementary sulfur. In this holophytic habit bacteria are unlike animals since animals which utilize solid foods do so by holozoic mechanisms, i.e., solid foods are brought directly within the confines of the body for digestion as well as assimilation. Though simple engulfment may occur the majority of animals possess a morphologically differentiated apparatus of digestion, a feature entirely absent in bacteria and typical plants. In addition, animals usually possess specialized apparatus for excretion, if only a contractile vacuole, structures which are entirely absent in bacteria. Plant cells lose water and water soluble materials by direct diffusion out of the cell into the external environment. Very few plant cells possess a contractile vacuole, and they have no means for the elimination of solid wastes.

3) Bacteria multiply by binary fission, the cell splitting along the transverse axis. With some exceptions animal cells divide by binary fission along the longitudinal axis.

4) Most species of bacteria and all of the so-called true bacteria possess a morphologically differentiated rigid cell wall located outside the confines of the plasma membrane and cytoplasm. By plasmolysis the cytoplasm of

both plant and bacterial cells may be retracted from the wall which retains its shape. The materials lending rigidity to the cell wall are like carbohydrates in their chemistry. On the other hand, the outermost differentiated structure of animal cells is usually the plasma or cytoplasmic semi-permeable membrane and is of a nitrogenous nature. In some unicellular animals the cell does have rigidity and fixity of shape because of the presence of a specialized wall called the *pellicle*. Yet in many of these cases, unlike the cell wall of plants, the cytoplasm will not retract from the pellicle on plasmolysis. The composition of the pellicle may be mucoprotein, tectin (pseudochitin), or even cellulose-like.

These criteria may be accepted as sufficient to determine the relation of bacteria to plants, but a difficulty arises since there are organisms, including some species ordinarily listed as bacteria, which possess characteristics associated with both kingdoms. Thus the photosynthetic flagellated euglena have been claimed as objects of study by both zoologists and botanists. It is only by looking at the bacteria as a group and weighing these properties in a statistical sense that it becomes logical to place them in the class *Schizomycetes* of the division *Thallophyta* of the plant kingdom. In the sixth edition of Bergey's Manual the bacteria are defined as follows:

"Class *Schizomycetes* Nägeli: Typically unicellular plants. Cells usually small, sometimes ultramicroscopic. Frequently motile. As in the closely related blue-green algae (Class *Schizophyceae*), the cells lack the definitely organized nucleus found in the cells of higher plants and animals. However, bodies containing chromatin which may represent simple nuclei are demonstrable in some cases. Individual cells may be spherical; or straight, curved or spiral rods. These cells may occur in regular or irregular masses or even in cysts. Where they remain attached to each other after cell division, they may form chains or even definite filaments. The latter may show some differentiation into holdfast cells and into motile or non-motile reproductive cells (conidia). Some grow as branching mycelial threads whose diameter is not greater than that of ordinary bacterial cells, i.e., about one micron. Some species produce pigments. The true purple and green bacteria possess pigments much like or related to the true chlorophylls of higher plants. These pigments have photosynthetic properties. The phycocyanin found in the blue-green algae does not occur in the *Schizomycetes*. Multiplication is typically by cell division. Endospores are formed by some species included in *Eubacteriales*. Sporocysts are found in *Myxobacteriales*. Ultramicroscopic reproductive bodies are found in *Borrelomycetaceae*. The bacteria are free-living, saprophytic, parasitic or even pathogenic. The latter types cause diseases of either plants or animals. Seven orders are recognized."

A critical study of this definition will reveal imperfections as a definition. The student should search for them. The use of numerous qualifying terms,



i.e., usually, some, may, makes for ambiguity in meaning and does not give as exclusive a definition as might be desirable. The description of characteristics is not always in positive terms, and when branching mycelial threads are described as not greater in diameter than that of ordinary bacterial cells the definition is in danger of being circular.

In the Manual the orders and suborders of the class *Schizomycetes* are given in the following key. A *key* is merely a table of salient characteristics of groups of organisms so arranged as to facilitate the identification of an unknown organism. As a diagnostic aid this particular key possesses a most unhappy deficiency, namely, the tendency to use negative characteristics:

A. Cells rigid, not flexuous. Motility by means of flagella or by a gliding movement.

1. Cells single, in chains or masses. Not branching and mycelial in character. Not arranged in filaments. Not acid-fast. Motility when present by means of flagella.

Order I. *Eubacteriales* Buchanan

- a. Do not possess photosynthetic pigments. Cells do not contain free sulphur.
- b. Not attached by a stalk. Do not deposit ferric hydroxide.

Suborder I. *Eubacteriineae*

- aa. Attached to substrate, usually by a stalk. Some deposit ferric hydroxide.

Suborder II. *Caulobacteriineae*

- bb. Possess photosynthetic chlorophyll-like pigments. Some cells contain free sulfur.

Suborder III. *Rhodobacteriineae*

2. Organisms forming elongated, usually branching and mycelial cells. Multiply by cell division, special spores, oidiospores and conidia. Sometimes acid-fast. Non-motile.

Order II. *Actinomycetales* Buchanan

3. Cells in filaments frequently enclosed in a tubular sheath with or without a deposit of ferric hydroxide. Sometimes attached. Motile flagellate and non-motile conidia. Filaments sometimes motile with a gliding movement. Cells sometimes contain free sulfur.

Order III. *Chlamydobacteriales* Buchanan

B. Cells flexuous, not rigid.

1. Cells elongate. Motility, by creeping on substrate.

Order IV. *Myxobacteriales* Jahn

2. Cells spiral. Motility, free swimming by flexion of cells.

Order V. *Spirochaetales* Buchanan

Supplements: Groups whose relationships are uncertain.

1. Obligate intracellular parasites or dependent directly on living cells.

- a. Not ultramicroscopic and only rarely filterable. More than 0.1 micron in diameter.

Group I. Order *Rickettsiales* Gieszczykiewicz

- aa. Usually ultramicroscopic and filterable. Except for certain pox viruses of animals and a few plant viruses, less than 0.1 micron in diameter.

Group II. Order *Virales* Breed, Murray, and Hitchens

2. Grow in cell-free culture media with the development of polymorphic structures including rings, globules, filaments and minute reproductive bodies (less than 0.3 micron in diameter).

Group III. Family *Borrelomycetaceae* Turner



A general difficulty encountered in constructing systems for the classification of bacteria is the paucity of evidence as to the evolutionary background or phylogeny of present day species. This lack makes classifications of bacteria based on natural relationships purely speculative attempts. Yet the scientific ideal is a phylogenetic classification which will do two things simultaneously; permit the identification of cultures with known organisms and indicate their natural relationships. Unfortunately the characteristics which indicate natural relationships are not always those most useful, that is, practical for diagnostic or determinative purposes. Bergey's Manual does not escape this dilemma.

The methodology for creating systems of classification can be either constructive or analytic. The constructive approach is one in which larger groupings are built from intensively studied smaller groups of individuals. Such an approach is the only one that can be used in the early development of taxonomic systems and is particularly suited to the determinative function of taxonomy. The early important systems of bacterial classification, those of Cohn (1872) and Migula (1900), were the result of the application of the constructive method and had only a determinative purpose. However, as knowledge of groups of organisms expands it becomes possible to build classifications by an analytical procedure. Some basic hypotheses of evolutionary development can be set up, and from the postulates chosen the organisms can be split into naturally related groups. These groups in turn are subdivided into still smaller and more closely related groups. The earliest attempt at a phylogenetic system for bacteria was that of Orla-Jensen (1909). This system may be discussed here to indicate the kind of difficulty often met in fulfilling the determinative purpose of taxonomy by a purely phylogenetic system.

Orla-Jensen postulated that the original and primitive bacteria were autotrophic. Ignoring the possibility of retrograde evolution, such a postulate would demand that all living autotrophic organisms be placed in one large group apart from those bacterial species utilizing organic molecules as food. Orla-Jensen also pointed out that cell shape was not necessarily a character which indicated genetic relationships. The evolution of surviving species has probably involved the transformation of cocci to rods and rods to cocci on a number of occasions. Thus, if among species with autotrophic and heterotrophic habits both cocci and rod shaped organisms happened to be represented, a readily perceived and useful determinative character, namely, cell shape, could not have maximum possible use in the identification of an unknown organism.

The reason for this particular inefficiency rests on the fact that employment of any system of classification for a determinative purpose requires the use of a key. In the usual practice the key is drawn directly from the system of classification to which it is applied. The key in such a case is obviously

limited to the characteristics used in building the groups of the particular system. A useful diagnostic character that possessed no value in indicating natural relationships would be missing in a key for a phylogenetic system, and to this extent the determinative function of taxonomy would be ill-served.

It has been suggested that a practical way to meet the difficulty would be to construct keys for the identification of organisms on purely diagnostic characteristics. As long as such a key made no claim to indicate phylogenetic relationships it need not be bound to the rigid and exclusive use of characters of phylogenetic significance. The advantage of such a key would rest on its practical utility for the ready location of an organism within a system. In phylogenetic classifications once an organism is located, its place within the system would indicate its natural relationships to other organisms. Skerman (1949) has constructed one such key to the genera of Bergey's Manual. It appears to have a more practical diagnostic use than the keys included in the Manual.

The phylogenetic relationships of the orders *Spirochaetales* and *Myxobacterales* to the so-called true bacteria, the *Eubacteriales*, is obscure. There is good reason to doubt the scientific wisdom of placing the rickettsiae, viruses, and *Borrelomycetaceae* in the class *Schizomycetes*. Closer relationships have been claimed between some of the blue-green algae and *Chlamydobacteriales* than between the latter and the *Eubacteriales*. As a matter of fact, the phylogenetic relationship of all the organisms placed within the order *Chlamydobacteriales* has been rightly questioned. There is good reason, then, to believe that a more natural system of classification would split the *Schizomycetes* into more numerous classes with the true bacteria constituting a single class.

In a thoughtful essay Stanier and van Niel (1941) have summarized the problem of the relationships of the bacteria and have discussed in detail some of the difficulties associated with the concept of the *Schizomycetes* according to Bergey's Manual. They have proposed a new kingdom, the *Monera*, to include all microorganisms without true nuclei, plastids, and sexual reproduction. The key they propose to the subdivisions of the *Monera* is as follows:

**Kingdom *Monera*:**

- A. Organisms photosynthetic with the evolution of oxygen and possessing the typical green plant chlorophylls, phycoerythrin, and sometimes phycoerythrin, and colorless, non-photosynthetic counterparts, clearly recognizable as such.

**Division I *Myxophyta***

- B. Organisms not so characterized.

**Division II *Schizomycetae***



- I. Unicellular or mycelial organisms with rigid cell walls. Motility, when present, by means of flagella. Endospores, cysts, or conidia may be formed.
  - a. Organisms photosynthetic but not producing oxygen.
    - Class 1 *Eubacteriae*
    - Order 1 *Rhodobacteriales*
  - b. Non-photosynthetic organisms.
    1. Unicellular.
      - Order 2 *Eubacteriales*
    2. Mycelial organisms.
      - Order 3 *Actinomycetales*
- II. Unicellular rod-shaped organisms, without rigid cell walls. Always creeping motility. Microcysts and fruiting bodies may be formed.
  - Class 2 *Myxobacteriae*
  - One order *Myxobacteriales*
- III. Unicellular, spiral organisms without rigid cell walls. Motility by means of an elastic axial filament or modified fibrillar membrane.
  - Class 3 *Spirochaetae*
  - One order *Spirochaetales*
- IV. A provisional appendix includes organisms not falling into the previous classes and whose natural relationships are entirely uncertain. Five families and seven genera are listed. The original authors do not make this suggestion, but obviously the rickettsiae and *Borrelomycetaceae* might be included as a mere matter of convenience. The virus group would remain outside the scope of this classification.

In suggesting how the class *Schizomycetes* might be split into a number of classes the above grouping makes a very valuable contribution. This course may well be adopted in the future by those responsible for Bergey's Manual and by bacteriologists in general. However, the suggestion of the creation of a new kingdom, *Monera*, requires critical consideration.

The difficulty associated with attempts to definitely assign all known microbes to either the plant or animal kingdom has suggested that the recognition of only two kingdoms is too simple a taxonomic device. It has been tempting to think in terms of a new kingdom to include these difficultly classified organisms. However, before an opinion can be formed as to the value of this scheme, it will be well to consider the basis for creating kingdoms.

All the evidence indicates that groups of organisms evolve by branching from preceding groups. For this reason biologists prefer a tree rather than a straight line for the diagrammatic illustration of the evolution of species. In agreement with this principle of evolution the recognition of two kingdoms, plant and animal, means that the course of evolution has been understood to be only along two major branches from an original stem. It is quite doubtful that the original forms from which the present-day species of plants and animals derived are still in existence. So there is no need to



create a new category, or super-kingdom, to include presently nonexistent and entirely unknown species of primordial organisms from which the plants and animals stem.

The creation of another kingdom must be rejected if it in any way implies a third major channel of evolution from the stem that gave rise to plants and animals. There is no convincing evidence for such a major evolutionary separation. However, to reject the creation of a third kingdom does not solve the problem of the classification of organisms of indefinite relations. Actually the problem is probably insoluble as long as the means for discovering the path of past evolution of these species remains unknown. Under the circumstances the problem should be recognized for what it is. We must wait for the flash of genius that may reveal the means for reaching a solution rather than conveniently settle the problem by the creation of an artificial kingdom.

It has been commonly stated that many of the organisms of indefinite phylogeny are intermediate forms between plants and animals, but it should be recalled that there is no unequivocal evidence for the present day occurrence of the original ancestors from which present day plants and animals developed. To call existing organisms intermediate is correct only if the implication is merely that these organisms have features in common with both plants and animals. The possession of these characteristics can be due, and in many cases probably is due, either to the loss of characteristics, or the evolutionary branching away from exclusively plant or animal features. Thus the plant that suffers a loss of synthetic capacity begins to resemble an animal in nutritional habit. The protozoan with a pellicle resembling cellulose and which ingests, or is parasitized by photosynthetic plant microbes, and learns to utilize the plant pigments for photosynthesis begins to appear as a plant. In these situations the organism is still related to the original parent kingdom in spite of its new-found and descriptively confusing characteristics. So it is inconsistent with the principles of a natural classification to put these organisms in a new kingdom though it may be logical to create within the plant and animal kingdoms new divisions for these species.

As a minimum task before any species can be truly said to be intermediate between plants and animals it will be necessary for someone to show that the organism has not developed by a course of evolution away from some ancestral true plant or animal form. We say this is a minimum task because the elimination of the possibility of convergent evolution is also necessary. *Convergent evolution* is any tendency of phylogenetically unrelated organisms to evolve in a common direction and is known to occur. For example, the whale has evolved in the direction of a fish-like form but, of course, is unrelated to any fish species.

Specific criticisms of the kingdom *Monera* as proposed by Stanier and van Niel were discussed in detail by Pringsheim in 1949. The definition of *Monera* is objectionable since it is based on purely negative characteristics, namely, the lack of typical nucleus, plastids, and sexual mode of reproduction. A more serious criticism is that the affinity of the blue-green algae (*Myxophyceae*) with the true bacteria is doubtful. Any resemblance between the blue-green algae and bacteria may actually be due to convergent evolution of phylogenetically unrelated organisms. In addition the relationship of the myxobacteria and spirochaetes is considered to be doubtful enough not to permit the placement of these organisms in the same division. Some evidence exists for considering particular species of myxobacteria and spirochaetes to be related to the *Myxophyceae* rather than the bacteria.

#### THE SPECIES CONCEPT IN BACTERIOLOGY

The definition of the term species might be thought to be a simple one and so basic as to have been an axiom fixed for all time at the birth of scientific biology. Yet the concept of what constitutes a species has proven to be elusive and has undergone changes coincident with the progress of biology. It was only after the theory of organic evolution found general acceptance and the modern science of genetics provided the theoretical basis for an understanding of the mechanisms of evolution that it has become possible to delineate the concept of species by means of definitions that show promise of becoming definitive. However, even if progress has been made in the definition of species the application of this definition to bacteria meets with serious obstacles. In the following discussion it is our aim to summarize the modern genetic concept of species and to consider the problem of species in bacteriology.

A definition of species that finds wide acceptance among students of taxonomy and genetics is the one adopted from Dobzhansky: *A species is a given stage of evolution at which actually or potentially interbreeding arrays of forms become segregated into two or more separate arrays which are physiologically incapable of interbreeding.*

This definition has the virtue of including the element of history or time in the species concept as well as the traditional concern with the common hereditary background of individuals composing the species at any given moment. Implicit in this definition are several important facts:

- 1) The species is a statistical concept and is defined not by a study of differences between individuals but by a study of differences between populations of individuals.

- 2) The definition focuses attention not on the differences that biologists have traditionally sought in separating species but on the gap that prevents interbreeding. For an understanding of species this definition directs the



search toward mechanisms that prevent interbreeding. These means of isolation can be environmental, geographic, morphological, psychological or physiological.

3) The species is a genetic concept since the species is a population capable of exchanging genetic material (chromosomes, genes) only within itself. Changes in the hereditary characteristics of the group are limited to the accumulation of mutations and chromosome changes in isolated stocks. It is only as accumulating changes result in infertility in crosses between the isolated and parent populations that new species arise. This conception is a productive one for it suggests means for experimental study of the evolution of species.

4) The species is a dynamic concept. At a given stage of history different groups will have traveled unequal lengths along the path toward speciation. Where the gap between isolated groups has not become complete, intermediates will be found that are not yet species and which the taxonomist cannot neatly catalogue. Recognition of this situation is valuable since it does not discourage the biologist from accepting the reality of species in spite of any inability to catalogue all groups of populations. The existence of the intermediate or indeterminate groups is no longer an obstacle to the concept of species. In fact these groups are confirmation of the dynamic quality of species and living proof of the fact of organic evolution.

5) The population constituting a species of sexually reproducing organisms possesses a characteristic genetic composition, for there is no gainsaying the probability that the morphological and physiological characteristics preventing the interbreeding of species must have a genetic basis. The inability of species to interbreed is a phenotypic expression of genotype.

With living species outside the laboratory it is not often possible and with extinct species it is impossible to determine the potential and actual limits of interbreeding of populations that would represent the species and subspecies boundaries. The practical means available to the taxonomist are limited, and as of old he still must make use of morphological characters in studying speciation. What then is the relation between the morphological and genetic concepts of species? The relation has been succinctly stated by Simpson whom we quote: "A morphological species is a group of individuals that resemble each other in most of their visible characters, sex for sex, and variety for variety, and such that adjacent local populations within the group differ only in variable characters that intergrade marginally. This morphological definition is merely a description of the usual result of the situation involved in the genetic definition. The morphological species is not a subjective or artificial concept, it is a real group that is taken as a sufficient approximation of the genetic species."

From the point of view of maintaining the integrity of the species and



of determining the evolution of the species there would be two kinds of morphological characters. There are those that help maintain the gap between species by affecting the mechanisms that prevent or influence interbreeding in any way and those neutral characters that play no role in maintaining the gap. Wherever practical the former characters should be given major consideration by the taxonomist employing morphological criteria for delineating species.

At the present time evidence for a sexual mode of reproduction has been found only for certain strains of a single species of bacteria (*Escherichia coli*). Thus the lack of a known sexual mode of reproduction does not permit a literal translation of the preceding definition of species to the bacteria. Strictly speaking, the bacteriologist works with a clone, a clone being a group of individuals of like hereditary constitution traceable through asexual reproduction to a single ancestral cell. In a population of asexually reproducing organisms there is no opportunity for interchange of hereditary material between individuals. There can only be direct transfer from one individual to its own daughter cells. The bacteriologist is thus dealing with organisms having a fixed gap in the interchange of genetic material. Each clone is a genetically isolated population, and in this sense a clone is equivalent to the species of the sexually reproducing organisms, since both are unable to pass genetic material to other populations. A logical conclusion, therefore, might be that if the term "species" is to be used by the bacteriologist there are an infinite number of bacterial species. The patience and energy of the bacteriologist in isolating clones would be the only limit to the number of species. This arrangement is impractical and, more important, it does not seem to correspond to the impression the experienced bacterial taxonomist has of the real situation in nature.

When large numbers of bacterial cultures isolated from natural sources are studied it becomes evident that groups exist with widely separated characteristics. One often observes clusters of clones with combinations of characters that occur with great frequency and other groups of clones with combinations of characters that occur rarely or not at all. This situation is exactly that expected if in nature there were related clones in various localities but an absence of a continuous spectrum of intermediate types between clones of different characteristics. This observation justifies considering the naturally occurring basic taxonomic unit for bacteria to be larger than the individual clone.

Though generally unstated, there is a guiding principle that the student of asexually reproducing organisms has accepted and used for the separation of the natural and genetically related groups or clones. The advantage of a formal statement of such a principle would be its power to suggest the theoretical and eventually the practical means of searching for the limits

of natural groups. The principle is that groups of clones composed of organisms containing the same genetic (chromosomes, genes) material are the natural, though not identical, equivalent to the species of the sexually reproducing organisms. Accepting this principle the diagnostic problem of bacterial taxonomy becomes one of determining the similarity of genetic material in different, experimentally isolated clones. Direct approaches would be the following:

- 1) In theory, identity in the structure and morphology of nuclear material would be expected in organisms from the same species. Actual use of this criterion has been made by biologists. However, it is an impractical diagnostic test with bacteria, for it is only in recent times that any worthy evidence has begun to accumulate concerning the possession by these organisms, of morphologically definable centers of genetic material. It is also well to recognize that cells with only one or a few chromosomes have greater opportunity to show gross similarity by mere chance alone than do cells with greater numbers of chromosomes.

- 2) One may suspect that the differences in the genetic material of different species are traceable ultimately to differences in the chemistry of chromosomes and genes. While this is yet an impractical criterion to use in taxonomy it is well to point out that this field offers unexplored opportunities to the combined efforts of biochemists, serologists, and taxonomists. It is conceivable that the isolation of the morphological units of heredity from cells and the study of their *in vitro* serological specificity will present unique opportunities for the determination of differences in genetic material associated with the recognizable taxonomic groups. At least, research along the suggested lines would begin to reveal how wide a difference in the chemistry of the nucleus and its chromosomes accompanies phenotypic variations.

Inasmuch as the aforementioned direct approaches are not practical at the present time, indirect means must be employed to study the genetic identity of clones. Again there are two possibilities worthy of consideration:

- 1) Mapping of the genes and their linkages. If clones have the same genetic constitution the genes controlling the phenotypic characters would be expected to exhibit the same location in chromosomes. However, once again we are confronted with a criterion of no present value in bacteriology, since the known methods of mapping depend on the capacity of organisms to reproduce sexually. The principle is a valid one, and it is hoped that methods can be invented for application to asexually reproducing organisms. We must wait upon the imagination and industry of skilled investigators to develop the necessary technics.

- 2) A statistical study of the numerical frequencies of various characters and combinations of characters of large series of clones isolated directly



from nature. The validity of this method rests on the finding that genetically related populations will have numerous phenotypic characters in common. This assumption seems to be true for all organisms and is independent of the mode of reproduction. By increasing the number of characters studied it should be possible to increase the confidence placed in any separation of groups arrived at by this method. In a rudimentary fashion the procedure has always been used by bacteriologists, but concrete formulation and theoretical development and illustration of the procedure we owe primarily to Andrewes and Hoarder (1906), the Winslows (1908), and Rahn (1929).

In asexually reproducing organisms any heritable variation that occurs persists by direct transmission to the progeny of the original single cell. Therefore, it is reasonable to conclude that after a sufficient number of generations all possible combinations of characters will occur in nature. The actual finding that all the possible characters do not occur indicates the existence of forces operating against the persistence of particular possible variants. These forces can be collectively labeled *selective forces*. In any natural environment different combinations of particular characters will have unequal survival values. Given sufficient time the flora persisting in an environment will be composed solely of the most successful genotypes, that is, the genotypes of superior competitive capacity.

Independent of the method of reproduction the phenomenon of gene mutation provides the variants on which selection pressures operate. The surviving and differing genotypes may then be considered the natural objects which the bacterial taxonomist seeks as species. In this context the important difference between species of sexually and asexually reproducing organisms lies in the fact that with the former the actual limits of interbreeding of populations are somewhat flexible obstacles that prevent the diffusion of successful genotypes, while in asexual organisms there is no possibility of diffusion.

#### THE METHODOLOGY OF BACTERIAL TAXONOMY

The bacteriologist, unlike most biologists, is severely limited in his ability to study his biological materials in their natural habitats. In general he must isolate cultures from natural sources and then study them in the laboratory. Hence the student should recall that it is the interplay of the environment and genotype which determines the actual phenotypic character of organisms. On this score the method of the bacteriologist is open to severe criticism. By capturing and caging organisms in the laboratory environment, which never exactly duplicates the natural environment, the phenotypic characteristic may change though the genotype remains constant. A specific genotype can have varied phenotypic expression depending upon the nature of the environment. The environment determines the



actual characteristics that will develop within the limited potentialities fixed by the genotype.

It must be recognized that often the bacteriologist is not describing the natural phenotype but the phenotype characteristic for the laboratory situation. Having no direct means of testing the identity of genotypes, two bacteriologists studying the same genotypes by analysis of the phenotypic characteristics under different sets of environmental conditions (these being different in unknown or uncontrolled ways) might well conclude erroneously that they were working with different organisms. Recognition of this weakness in methodology should result in a search for remedies. Among others, the following suggestions deserve consideration since they would help ensure comparability of the results of investigations separated in time and space. Their adoption would help minimize conflicts in interpretation and in the division of taxonomic groups.

- 1) The laboratory environment should simulate the natural environment as closely as possible. This is particularly necessary if we are to understand the characteristics of bacteria as they occur in nature. It is nice to know what bacteria look like and how they behave in invented environments, but the aim of taxonomy is to determine the characteristics of naturally occurring populations.

- 2) Because it will not be possible in most cases to duplicate the natural environment, cultures should be studied after only one or a few transfers from the original isolation. Thus the possibility of preserving the genotype is increased. In a new environment mutations will have different survival values than were possessed in the natural environment, and given a sufficient number of generations the mutants may replace the parent population. Fortunately, techniques such as lyophilization are available for the long term preservation of cultures; thus cultures can be preserved immediately after isolation. This procedure enables the simultaneous study of large numbers of clones isolated at different times after the same number of transfers from their natural habitat. Of course the possibility that the preservation method may have better survival value for particular mutants cannot be ignored. So preservation while minimizing the opportunity for change does not necessarily guarantee that a culture is absolutely identical to the original population.

- 3) Finally, the bacteriologist engaged in a taxonomic investigation should define the conditions of his study in detail and with the utmost precision.

The perceptive student will recognize that the foregoing discussion still leaves an important question unanswered. While groups of both sexual and asexual organisms present arrays of characteristics that permit a logical hierarchical arrangement of taxa,<sup>1</sup> the question must be raised as to how

<sup>1</sup> *Taxa* are taxonomic groups. Singular is *taxon*.

much diversity in genotype is permissible in the basic groups of the system labeled as species. There are no accepted criteria for defining boundaries of genetically related groups or "species" of asexually reproducing organisms. In sexually reproducing organisms the test of interbreeding is available in theory if not in practice.

In this regard it must be emphasized that the species of sexual organisms are not the creation of the taxonomist, they are derived by the taxonomist from a fact of nature. The species population is an actually or potentially interbreeding population. The presence of observable genotypic variations within this population does not disturb the definition of species. These variations have no taxonomic significance for the population identified as a species as long as they do not affect capacity for interbreeding.

On the other hand, asexual species are arbitrary creations unless it were agreed that the term species must be applied to all clones showing any genotypic variation whatsoever, even if this be only a single gene mutation. There are few bacteriologists who would be willing to limit the term "species" to these natural groups since it would multiply tremendously the numbers of so-called species to be recognized. In any case, it is valuable to understand that if bacteriologists are unwilling to label each genotypic variation a new species, then any other criterion for grouping of bacteria into so-called species, though it may have an admitted convenience, is a purely arbitrary exercise.

Since there remain undiscovered any natural boundaries for delimiting the genotypic variations to be placed within a single species of bacteria, can any sensible, even if arbitrary, criteria, be universally adopted by bacteriologists? The literature does not reveal any systematic exploration of this problem, and this task awaits solution in the future. However, the following possibilities are worthy of thought:

- 1) Groups varying by but a single gene mutation from a described species should be placed in the species.

- 2) Anyone describing a new species has a moral obligation to study the naturally occurring variants of the group and to state which of these variants are considered to be still within the species limits. This requires that the investigator be familiar with the concept of the *type culture*. This principle as stated in Bergey's Manual is: A species of bacterium is the type culture or specimen together with all other cultures or specimens regarded by an investigator as sufficiently like the type to be grouped with it.

- 3) The person describing a new species should deposit a specimen with a type culture collection. In the United States this would be with the American Type Culture Collection, Washington, D. C. The investigator should also maintain his own specimens, preferably as viable resting cul-



tures, and should be prepared to supply other investigators with transfers and with a detailed statement of the methods of study used in the original description.

#### CHARACTERISTICS OF BACTERIA EMPLOYED IN THEIR CLASSIFICATION

Traditionally, taxonomic systems have drawn almost exclusively upon morphological characters for the designation of taxa, and the same tendency has existed to a lesser extent in bacterial taxonomy. Since bacteria present relatively few visual morphological features for study, other possibilities, and particularly physiological characters, have not been ignored. However, when the opportunity has presented itself, morphological criteria have been preferred to other characteristics. Thus the major subdivisions of bacteria of the present widely used classifications have been based on differences in morphology. It is in species designations that physiological features have had the more prominent use. For a natural system of classification this procedure presupposes that evolution has had primarily a morphological emphasis. The arguments in support of the reasonableness of this hypothesis are the following:

- 1) At the cellular level there is a fundamental similarity in the metabolism of all known living organisms. This finding is one of the chief contributions made to date by studies in comparative biochemistry.

- 2) The physiologically related groups remain together in a morphological system, whereas in physiological systems organisms with greatly differing morphology are often brought together.

- 3) Physiological differences must have a morphological basis, even if these are below the limits of vision, and presumably, refinements in instrumentation would reveal the differences in morphology that accompany the physiological differences.

- 4) Taxonomic analysis must be carried as far as nature itself is subdivided, and organisms seem to be more varied morphologically than they are physiologically. This can be best illustrated by the fact that enzymes performing the same physiological or metabolic function in various organisms are often antigenically different. This observation is well established with the hemoglobins and catalases of various animals, but this type of analysis is relatively undeveloped in bacteriology since the isolation of purified enzymes from different bacteria is still in a rudimentary stage of development. However, there is no reason to believe that a similar situation does not exist among bacteria. In any event, it would certainly be instructive to know, for example, whether *Escherichia coli* and *Aerobacter aerogenes*, organisms separated on the basis of other characteristics, possess antigenically identical or dissimilar lactases. Such differences in antigenicity are akin



to differences in morphology since they are reflections of varied chemical structure.

The consideration of the properties of bacteria used in taxonomy may now be completed by discussing briefly some specific characters:

### *Unit Characters*

Wherever possible unit characters are employed by the taxonomist in his descriptions. They are those characters without any intermediate condition, say, for example, the possession of endospores or the fermentation of a sugar. An organism either does or does not possess endospores or ferment a test sugar. Non-unitary characters, such as the size of the cell or the quantity of acid produced from a given substrate, often have a logical value but are not as convenient to employ for classification, especially when the taxonomic purpose is diagnosis.

### *Pleiotropism*

When a single gene affects several of the characteristics of an organism the phenomenon is known as pleiotropism. It is important for the bacteriologist to be acquainted with this phenomenon since otherwise it may lend a false value to the data of the numerical frequency procedure for distinguishing groups of bacteria. It will be recalled that in this procedure characters are tabulated for statistical treatment, and groups are distinguished by the frequency of isolation of clones from natural sources. Since phenotypic characters are actually studied when it is really the genotypes that are being sought, it is wrong to give the same weight to each of a series of characters all controlled by the same gene as is given to other characteristics each of which is under the immediate control of different single genes. Pleiotropism is known to occur in bacteria. It is probably in large part responsible for the phenomena of dissociation.

### *Antigenic Characteristics*

At the molecular level differences among organisms must have their basis in variations in chemical composition. The antigen-antibody reaction is a delicate, and often the only, means for detecting differences among antigens and haptenes. Understandably the study of antigenic differences between organisms has been actively pursued, and there has been a gratifying correlation of serological phylogenetic trees of plants and animals with phylogenetic systems built on other bases. These studies point to the existence of two systems of antigenic specificity in nature: (1) protein specificity which has undergone a gradual change in the course of evolution and (2) hapten specificity which has been characterized by sudden changes not

linked by intermediate stages. The haptenes studied have usually been polysaccharides.

Apart from the proteins which are organ specific (e.g., eye lens protein) different organisms have antigenically dissimilar proteins, which phenomenon, as has already been pointed out, even extends to differences in enzymes performing the same functions. With some haptenes of a carbohydrate nature there have been described widespread distributions in organisms phylogenetically far removed. An example of this type is the occurrence of human blood group A substance in other animals and in the type 14 pneumococcus. These findings point out that for taxonomic purposes it is more valuable to study the antigenicity of proteins or of the protein moiety of conjugated proteins than any other materials. To obtain the most significant and easily interpreted data, taxonomic research utilizing the antigen-antibody reaction should deal with purified or characterized mixtures of protein rather than with whole cells.

In bacteriology antigenic studies have been numerous and of proven value in certain groups. It is probable that the full power of the serological tool still remains to be utilized since most studies have employed whole cells rather than purified materials. In one aspect antigenic analysis has been discouraging since it has multiplied the task of taxonomy by revealing an increasing number of differences within groups designated as species by other criteria. It is also true that antigenic variations occur and that these in many cases are the result of single gene mutation. However, since the task of science is to understand nature, analysis must follow wherever nature leads, even if this reveals an ever increasing complexity and multiplicity of forms.

### *Physiological Characters*

The relative paucity of visual morphological features of bacteria has led to the extensive consideration of physiological characteristics for diagnostic purposes. This approach represents a break with the traditional and exclusive use of morphological characters by other biologists. Still, the break is not complete since the systems of bacteriology generally limit the use of physiological characteristics to the separation of varieties, species, and genera. A notable exception is the classification proposed by Orla-Jensen in which the major subdivisions were based on nutritional requirements and end products of metabolism.

The employment of certain physiological characters should be done with circumspection because of the remarkable adaptive powers exhibited by many bacteria. Although, if information is gathered on the adaptive capacity of organisms being studied and the conditions of the study are reproducible, there is no objection to the use of such characteristics as limits of



growth determined by temperature, pH, oxidation-reduction potential, surface tension, and osmotic pressure.

The energetics of the organism, the paths of intermediary metabolism, and the end products of catabolism are all important and characteristic features of bacterial metabolism. They are worthy of consideration in diagnostic bacteriology though their significance in establishing phylogenetic relationships is often obscure. Differences in the hydrolytic capacity of bacteria such as the ability to digest casein or to utilize disaccharides are of minor metabolic significance and are often subject to single gene mutation. These characteristics might have value for distinguishing varieties or species but would seem to have no real value for differentiating higher taxonomic groups in natural systems of classification.

Pathogenicity is a physiological characteristic that has deservedly fallen into disrepute as a means for distinguishing bacteria. The reasons for this change of emphasis are several: (1) pathogenicity varies with a given strain, (2) it may be readily lost on laboratory culture, (3) it is a relative and non-unitary characteristic. There is a spectrum in the differences of pathogenicity of the strains of a given organism so that the line between pathogenicity and non-pathogenicity is arbitrary, (4) it varies with the choice of and variations (usually unknown) in the host. As an absolute statement it is incorrect to say that an organism is not pathogenic, for pathogenicity is never tested in a large and varied group of host organisms. The confusion that can result from the taxonomic use of pathogenicity is illustrated by the case of *Pseudomonas pyocyaneus*, an animal pathogen also capable of causing a natural infection of plant species. As a plant pathogen it was for many years called *Pseudomonas polycolor* and not suspected to be the same organism known to animal pathologists as *Pseudomonas pyocyaneus*.

### THE ORIGIN AND EVOLUTION OF BACTERIA

The origin of life and the nature of its first forms is a challenging scientific problem. The small size, relative morphological simplicity, and autotrophic habits of certain bacterial organisms suggested to the early bacteriologists that bacteria might be the primordial forms of life. Hence the problem of the origin of life has often been considered to be the problem of the origin of bacteria. It is no mere coincidence that in common parlance bacteria are called germs, for the etymological significance of the word germ lies in its derivation from the Latin *germes* or *germinis* meaning sprout, that from which anything springs.

As early as 1865 it was postulated that germs resistant to the rigors of interstellar space and carried by cosmic dust and meteorites seeded the earth with the first forms of life. The presumed inorganic nature of the



sterile surface of the earth and the remarkable resistance of bacteria, and particularly spores, to cold and desiccation suggested that these original germs might be autotrophic bacteria. Arrhenius, the great physical chemist, about fifty years ago calculated the force necessary for the transfer of particles among celestial bodies and showed the pressure of light to be a sufficient agency.

A direct experimental attack on this hypothesis has been made by a study of the bacteriology of meteorites, and curiously enough bacteria have been found in meteorites. Inasmuch as the bacteria isolated have been identical to forms already present in soil, most bacteriologists assume that their presence in meteorites indicates a subtle kind of contamination following the arrival of the meteorite on earth. This attitude hardly seems a conclusive objection since it is quite possible that organisms capable of both interstellar transfer and a terrestrial existence might have persisted as living species from their first introduction to the earth. Then again, contamination of the earth's surface by meteorites is a continuous and everyday occurrence. Thus at any time the bacteria of meteorites might normally be found as a part of the soil flora. Neither the mere finding of bacteria in meteorites nor their identity with soil types is definitive proof or disproof of the hypothesis, so as far as this type of evidence goes the question remains open.

A more serious objection to the seeding of the earth by living forms from interstellar space is the gamut of high energy radiations to which such organisms would be subject during their travels in space. It is doubtful that any unprotected organisms could resist for even a short time the radiations with which they would be bombarded once outside the protective shields of planetary atmospheres. Even organisms trapped within the interiors of meteorites are not well shielded from the lethal high energy radiations of interstellar space.

A more fundamental question to ask is whether or not meteorites have had their origin from astronomic bodies supporting life. If this question could be settled first it would give a fresh and more convincing aspect to the worth of speculations on methods of transfer through space and hence to hypotheses of the extra-terrestrial origin of life on the earth. With the rapid advances being made in the knowledge and analysis of the isotopes of the elements it should finally be possible to determine whether the composition of meteorites includes materials, particularly carbon, of probable organic origin.

A philosophical objection to the postulated extraterrestrial origin of life has been that it merely begs the question by transferring the problem to some other place in space. While this is correct the extraterrestrial origin of life is still a vital and real problem. It is important to know whether in

fact life did or did not originate on the earth even if the answer to this question does not contribute to knowledge of the fundamental processes and events leading to the development of life from an antecedent sterile universe.

#### THE HETEROTROPH HYPOTHESIS

The progress of comparative biochemistry has revealed that metabolic complexity is directly associated with the food habits of organisms. The fewer and simpler are the foods required by organisms, the more numerous are the enzymes possessed by the organism and the lengthier are the metabolic paths leading from raw materials to final syntheses of cellular constituents. With this knowledge has come the inescapable conclusion that the first living forms could not be autotrophic organisms. As simpler organisms possessing fewer enzyme systems the heterotrophic organism must have preceded the autotroph. The heterotroph hypothesis of the nature of primordial organisms has therefore replaced the older autotroph hypothesis, and as a necessary corollary it has required the postulation of a long period of synthesis of organic compounds and the appearance of colloidal systems prior to the beginnings of life. This view and the possibilities for syntheses of organic compounds in an originally inorganic geochemical environment are thoroughly discussed by Oparin in his *The Origin of Life*.

The synthesis of organic compounds in a universe free of living organisms could go on progressively and undisturbed, but once living forms originated these would be simple heterotrophic forms which drew upon the stores of organic compounds accumulated over eons of time. As these food stocks were utilized and decreased, environmental demands were made on heterotrophs to adapt themselves to new food sources, namely, the decomposition products of their own metabolism and autolysis. The origin of life spelled the eventual depletion of the organic compounds synthesized from inorganic sources in a sterile universe. Darwin was apparently the first person to recognize that the origin of life meant the ultimate destruction of the chemical environment in which it developed. Furthermore, the origin of life could only take place once since living forms destroyed the environment that gave them birth. With the loss of the original types of food the primordial forms of life were inevitably replaced by their descendants.

The heterotroph hypothesis means that known bacteria cannot have been primordial forms. Bacteria have their place in, and are completely dependent upon, the cyclic biological processes which replace raw materials as they are consumed. The autotrophs developed from heterotrophs by the acquisition of new synthetic powers, and they too have taken their place in maintaining the dynamic balance of cyclic utilization and deposition of elements inherent in the total biological picture of life.



## EVOLUTION OF BACTERIA

The evolution of bacteria is a matter of conjecture. Two of the most powerful tools for studying the evolution of species are unavailable in bacteriology, namely the study of ontogeny and paleontology. There have been claims of the discovery of fossil bacteria. While there is no doubt that sedimentary rocks do sometimes contain objects which appear to be bacteria, the mere similarity of appearance of microscopic objects to bacteria does not establish their identity. Such a demonstration would be more convincing if acceptable hypotheses could be developed to explain how soft-bodied, microscopic organisms like bacteria might be fossilized.

A more analytical procedure is deduction of the presence of bacteria at certain periods of geologic time from a knowledge of the role of bacteria in the biological origin of certain sediments and mineral deposits. In addition, comprehensive studies of the types of bacteria serving as food for other organisms, particularly hard-shelled protozoa, would permit the deduction of the coincident presence of particular kinds of bacteria in those sediments harboring the fossils of these bacterial predators. These possibilities have not been thoroughly exploited in published studies. An added incentive for such studies resides in the additional possibility of suggesting something about the physiological nature of bacteria at different periods of geological time.

If one accepts as authentic the described fossils of bacteria, the first appearance of bacteria is in Algonkian sediments of the pre-Cambrian era. Additional and better evidence of bacteria in pre-Cambrian times are iron ore and limestone deposits of presumed bacterial origin in the most ancient pre-Cambrian sediments of the Archean age. These data would place the origin of bacteria at not less than one billion years ago. The most ancient fossils, also pre-Cambrian, for which any reasonable evidence based on the  $C^{12}/C^{13}$  ratio attests to their authenticity are those of an algae, *Corycium enigmaticum*.

A rather controversial claim has been made for the isolation of prehistoric living bacteria in rocks of pre-Cambrian times and in the coal deposits of more recent origin. Independently of the problem of how individual organisms could survive, even in a state of suspended animation, the vicissitudes of tens and hundreds of millions of years of geological stress and strain, a difficulty with these claims lies in the inability to conclusively establish that recent contamination by percolation of water is not responsible for the occurrence of these bacteria. Since the organisms isolated are not new types the problem of contamination is a real one and must be dealt with in a definitive way. If contamination could be eliminated as an explanation of their occurrence in ancient strata, their similarity to present day forms would not be an insuperable barrier to accepting their authenti-



city as persisting ancient species. A number of prehistoric species of both plants and animals are known to have survived into modern times, the horse-shoe crab being the most notable example, its existence as a species being of the order of two hundred million years. With this situation in mind it is conceivable that species as adaptable as bacteria may have survived for even longer periods of time.

Starting with the commonly accepted notion that the first bacteria had to be cocci, Kluver and van Neil have presented a hypothetical picture of the evolution of the true bacteria. They have also showed how a phylogene-

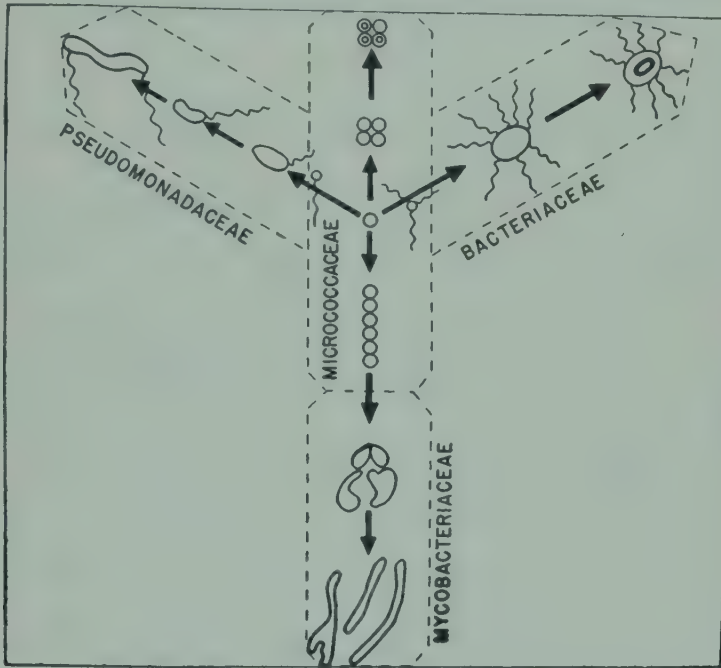


FIG. 1. A hypothetical scheme of the evolution of the bacteria taken from Kluver and van Niel (1936).

tic system of classification might be built upon the presumed paths of evolution. A summary of their concepts is illustrated in Figure 1. Students should consult the original paper for details.

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## CHAPTER III

# General Properties of Bacteria

### THE CELL THEORY AND BACTERIA

On the strength of repeated usage the individual bacterial organism is invariably referred to as a bacterial cell. As a matter of convenience this custom is justifiable, but still it will be worth critical examination in the light of what the appellation *cell* implies.

Following the invention of the means for the study of the microscopic structure of animals and plants there developed the knowledge that the overwhelming number of macroscopic living organisms were composed of divisible microscopic units of protoplasm. These units were labeled cells. The idea that cells were the primary units of organization of protoplasm developed gradually and finally found formal expression as the *cell theory* usually associated with the names of Schleiden and Schwann. The cell is defined as a mass of protoplasm containing a nucleus, both protoplasm and nucleus arising through the division of the corresponding elements of a pre-existing cell. The theory regards the cell as the cause of organization and differentiation. The evolution of the multicellular organism is viewed as the result of the aggregation of originally independent unicellular organisms. Microbes without cellular differentiation are thought of as single cells equivalent to the individual cells of multicellular organisms.

The fact remains that the cell theory is not necessarily an accurate representation of the actual biological situation. The dogma of the exclusive origin of cells from preexisting cells, *omnis cellula e cellula*, is challenged by the existence of organisms in which cells have their origin in a multinucleate or plasmodial mass. Then there are the *coenocytic* organisms like the *Phycomycetes*, fungi composed of a mass of protoplasm that is multinucleate and not subdivided into cells. Yet these organisms have a high degree of structural differentiation and produce enormous masses of visible growth under suitably nutritious conditions. Even in typically multicellular organisms there may exist tissues like muscle fibers that are masses of protoplasm called *syncytia* which are not subdivided into cells. Observations of these kinds of protoplasmic organization are difficult to interpret in terms of the cell theory and have led to the origin of the *protoplasm doctrine* or the *organismal concept*.

The protoplasm or organismal concept specifies the units of biological organization to be protoplasmic masses. Division into cells is not necessary

for growth or differentiation. Cells are accessory in metazoa. They are the result, not the cause of organization and differentiation. Evolution has occurred not as a result of the aggregation of individual cells into a multicellular republic but by differentiation of protoplasm. Thus microbes such as protozoa, algae, and bacteria are regarded as homologous with whole multicellular organisms, not with the cells of those organisms. Differentiation by these microbes is by specialization of internal regions. In the protozoa these specialized regions are the readily discerned organellae. According to this view an organism is any individual constituted to carry on the processes of life. These individuals may be *cellular* or *acellular*. If from habit we continue to speak in the succeeding pages of individual bacteria as bacterial cells, it is without any implication that we accept the cell theory as preferable to the protoplasm doctrine. To speak of the bacterial *body* rather than the bacterial *cell* would be more satisfactory.

The true bacteria are morphologically simple and undifferentiated acellular organisms with rigid walls. Apart from the order *Actinomycetales* they occur without true branching or have only a rudimentary tendency to form a mycelium. Reproduction is asexual by means of transverse fission. In species of actinomycetes the protoplasmic mass may grow indefinitely without subdivision into new individuals. The resultant mycelium may persist or eventually fragment into segments.

### SIZE

When bacteria are observed directly, four obvious characteristics are easily noted: size, shape, grouping or arrangement of individuals, and motility. The most distinctive feature of the appearance of bacteria is their small size. The smallest known species approach the limits of resolution of the light microscope. While bacteria as large as five micra in width are known, and species of the *Chlamydothales* such as *Beggiatoa mirabilis* may be as much as 40 micra in width, most of the eubacteria fall in the narrow range of sizes from 0.2 to 1.5 micra in diameter. These dimensions are noteworthy when compared with the magnitude of the cells of higher animals and plants (Table 1). Mitochondria alone, just one of the many constituents of the cytoplasm of animal cells, are of the same size as most bacteria. The thickness of the nuclear membrane of animal and plant cells is often of the order of one micron while chromosomes are anywhere from 0.2 to 50 micra in diameter.

The small size of the bacteria presents the investigator with unique problems. The experiences of biologists with larger organisms and the points of view to which they have led are not always directly transferable to the study and understanding of the bacteria. Special techniques have been needed in order to deal with these minute organisms. While other biologists

TABLE 1  
Comparison of the approximate dimensions of some small particles

MATERIAL	RADIUS* $\mu$	VOLUME $\mu^3$	SURFACE $\mu^2$	SURFACE VOLUME $\mu^{-1}$	DENSITY $\text{gm./cm.}^3$	PARTICLE WEIGHT $\text{gm.}$	PARTICLES	MOLE WEIGHT $\text{gm./mole}$	SURFACE AREA/ CM. $\mu^2$
<i>Valonia ventricosa</i> † Sphere 2 mm. in di- ameter	25000	$6.5 \times 10^{13}$	$7.8 \times 10^9$	$1.2 \times 10^{-4}$	1.05 (?)	68	0.015	$6 \times 10^{25}$	$1.2 \times 10^8$
<i>Saccharomyces cerevisiae</i> . . . . .	1000	$4.2 \times 10^9$	$1.3 \times 10^7$	$3.1 \times 10^{-3}$	1.0	$4.2 \times 10^{-3}$	240	$2.5 \times 10^{21}$	$3.1 \times 10^9$
<i>Escherichia coli</i> <i>E. coli</i> phage . . . . .	3 0.5 0.04	110 0.52 $2.5 \times 10^{-4}$	110 3.1 0.02	1 6 80	1.07 1.07 1.5	$1.2 \times 10^{-10}$ $5.6 \times 10^{-13}$ $3.3 \times 10^{-16}$	$8.3 \times 10^9$ $1.8 \times 10^{12}$ $3 \times 10^{15}$	$7.2 \times 10^{13}$ $3.3 \times 10^{11}$ $2 \times 10^8$	$9.1 \times 10^{11}$ $5.6 \times 10^{12}$ $6 \times 10^{13}$
<i>Helix pomatia</i> hemo- cyanin Catalase Ribonuclease	0.013 0.009 0.0015	$9.2 \times 10^{-6}$ $3 \times 10^{-7}$ $1.4 \times 10^{-8}$	$2.1 \times 10^{-3}$ $1 \times 10^{-4}$ $2.8 \times 10^{-5}$	240 330 2000	1.4 1.4 1.4	$1.1 \times 10^{-17}$ $4.2 \times 10^{-19}$ $2.1 \times 10^{-20}$	$9.0 \times 10^{16}$ $2.4 \times 10^{18}$ $4.7 \times 10^{19}$	$6.7 \times 10^6$ $2.5 \times 10^5$ 12,700	$1.9 \times 10^{14}$ $2.4 \times 10^{14}$ $1.3 \times 10^{15}$
Sucrose	$4.4 \times 10^{-4}$	$3.5 \times 10^{-10}$	$2.4 \times 10^{-6}$	6900	1.6	$5.6 \times 10^{-22}$	$1.8 \times 10^{21}$	342	$4.3 \times 10^{15}$
Water (Monomer)	$1.9 \times 10^{-4}$	$3.7 \times 10^{-11}$	$4.5 \times 10^{-7}$	15,000	1.0	$3.0 \times 10^{-23}$	$3.3 \times 10^{22}$	18	$1.5 \times 10^{16}$

\* For the sake of simplicity, all particles are assumed to be spherical.  
† Largest unicellular alga.



have been able to investigate the individual organism as such, the microbiologist has almost always been forced to deal with populations, the mass of microbes being easier to manipulate than the individuals. Population phenomena have characteristics of their own apart from the properties of the individuals composing the population, and since the bacteriologist is almost always the student of the many he tends to ignore the trees for the forest. On the contrary, students of other forms of life may more readily separate and understand the phenomena of both the individual organism and the mass.

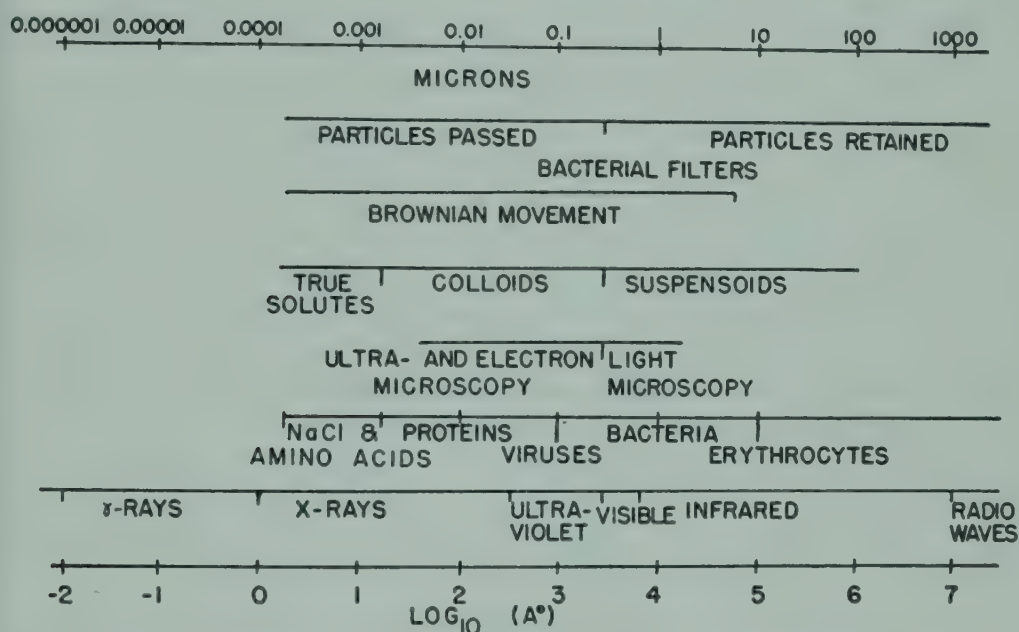


FIG. 2. Summarizing the relative sizes of various objects; dimensions are recorded at the top in  $\mu$  (1 mm. =  $10^3\mu$ ) and at the bottom in Ångstroms (1 mm. =  $10^7\text{Å}$ ). The radio region extends beyond  $10^{13}\text{Å}$  (1 kilometer).

The dimensions of bacteria place them at the upper fringe of size of colloidal matter (see fig. 2). Bacteria may be thought of as hydrophilic colloids and treated as such. Thus bacteria suspended in liquids will exhibit Brownian movement, scatter light by the Tyndall effect, and increase the viscosity of the suspending medium. In the case of the latter property the curious and unexplained observation has been made that a given suspension of bacteria will be at a maximum viscosity at the pH of the isoelectric point of the bacteria. Ordinarily the viscosity of sols and protein solutions will be at a minimum near or at the isoelectric point. Bacteria act as electrically charged colloidal particles. They will migrate in an electric field in a direction and at a rate determined by the pH, density, and viscosity of the suspending medium and by the potential difference across the electric field.

Individual bacteria will be agglutinated, and the stability of suspensions is dependent upon the quantities and nature of salts and dehydrating agents added.

The size of particles has a great effect on their behavior in any system. It will be evident from Figure 2 that particles in different size ranges are very different in their response to various treatments and to physical forces. From the consideration of Stokes' Law (see page 49) it will be seen that the radius is a key factor in the sedimentation of particles; the smaller the object, the lower is its rate of settling. For quite small particles Brownian movement becomes so powerful a dispersing force that these particles do not separate from the system under ordinary conditions.

While the radius has an important effect, other geometric features such as the volume and surface of the particle are involved in other ways. In the living organisms of small size which do not engulf their food materials, diffusion controls the rate of transfer of metabolites into the cell and the elimination of waste products. Growth at the rates observed for bacteria would be impossible for larger organisms depending solely upon diffusion. In the very small organism not only is the distance to be traversed quite short, but also the surface per unit of body weight across which the transfers are made is extremely large. Both factors are vital. See Table 1 for the radius-surface-volume data of some sample materials.

One would expect that cells smaller even than *Escherichia coli* might be able to grow most rapidly because they would have a larger surface-volume ratio, and metabolites could reach the interior more quickly. However, there seems to be a lower limit below which respiring, multiplying particles do not exist. This limit may be set by the volume requirements needed to contain the enzymes for growth, respiration, and reproduction. The enzymes, of course, are of considerable size and a large variety seems to be necessary for the operations involved in the phenomenon called life.

In this connection it is of interest to estimate the possible enzyme-protein content of a bacterium. *Micrococcus lysodeikticus* when dried has a weight of about  $2.5 \times 10^{-13}$  grams per bacterium. Of this weight 70 per cent is protein. Assuming that all the protein (even nucleoprotein) is enzymic and has an average molecular weight of  $10^5$  gm/mole, a maximum of approximately a million such molecules are present in a cell. Hence a million kinds of enzymes would be the upper limit. Although this figure seems fairly reasonable for the total number of protein molecules, it is probably too high as an index of the possible varieties of enzymes. In the first place while some proteins are considerably less than  $10^5$  in molecular weight, others are much higher and would have a more pronounced effect on the average molecular weight. Also there may be a large fraction of the protein which has no enzymatic activity. It is true that such material may be genetically

important, but it may consist of only a small number of kinds of proteins. Finally, many enzymes definitely are present in quantities of many molecules per cell.

*Micrococcus lysodeikticus*, for instance, may contain 1 per cent or more of catalase in the dried cells. This enzyme has been isolated from the organism, crystallized and studied. Its molecular weight is  $2.5 \times 10^5$ , and 4000 or more molecules of catalase are therefore present in each organism. If all the enzymes were present in this amount, there could be about 250 different kinds. However, since most enzymes probably are rather less abundant, this value represents a lower limit, and the actual number will lie somewhere between 250 and 1,000,000, perhaps between 1,000 and 10,000. In any case an infinite variety of enzymes does not seem possible at any given time although some enzymes may be converted into others by the process of enzyme adaptation if the environment is altered, thereby permitting added variety as the need arises.

The size of a bacterium is inherent in the nature of the species; it is genetically controlled. Under a given set of environmental circumstances the mean size of a population of a given strain and species is fixed although the mean size may shift with the age of the population. As a rule bacterial populations in the early stages of cultural development are composed of cells larger than those existing beyond the stage of maximum rate of growth of the population. Diphtheroids are believed to be an exception to this rule. In addition to age environmental factors such as the temperature, interfacial tension, and osmotic pressure are also important in determining the mean size of the bacteria in a population.

### SHAPE

In general three large morphological groups are recognized among the bacteria. These are:

	NAME	
	Singular	Plural
Sphere or ovoid.....	coccus	cocci
Rod or cylindrical.....	bacillus	bacilli
Spiral or corkscrew.....	vibrio	vibrios
	spirillum	spirilla

The shape of bacterial organisms is not immutable, for age and environment are important determinants of shape. Organisms as small as bacteria are subjected to enormous surface forces. Since the free energy of a system tends toward a minimum, flexible or plastic bodies will tend to assume the



TABLE 2  
*Comparison of the composition of liver cells with those of a typical bacterium*  
All data are only approximate since the exact values vary with the state of the cells and the methods of assaying.

	ESTI- MATED AVER- AGE MOLEC- ULAR WEIGHT	LIVER CELL				<i>Escherichia coli</i>			
		Dimensions employed	Wet weight	Molecules per cell	Relative numbers of molecules	Dimensions employed	Wet weight	Molecules per cell	Relative numbers of molecules
Size— $\mu$ .....		25	%			$1 \times 1 \times 2$	%		
Volume— $\mu^3$ .....		8000				1.5			
Density—gm., cm. <sup>3</sup> .....		1.1				1.1			
Weight—gm. cell.....		$8.8 \times 10^{-9}$				$1.6 \times 10^{-12}$			
Water.....	18		85	$2.5 \times 10^{14}$	50,000		75	$4 \times 10^{10}$	25,000
Protein.....	$10^5$		10	$5 \times 10^9$	1		17	$1.5 \times 10^6$	1
Lipids.....	700		2	$1.5 \times 10^{11}$	30		2	$3 \times 10^7$	20
Other organic.....	250		1.5	$3 \times 10^{11}$	60		4	$1.5 \times 10^8$	100
Inorganic.....	50		1.5	$1.5 \times 10^{12}$	300		2	$4 \times 10^8$	250

smallest possible surface area. For a given volume the smallest surface area is that of a sphere. From these considerations it might be expected that organisms as small as bacteria would be spherical. Yet among bacterial shapes the perfect sphere is the exception rather than the rule. This observation can only be accounted for by postulating the existence of a force counteracting the interfacial tension. The counteraction has its morphological origin in a rigid outer cellular structure, the demonstrable cell wall of bacteria.

### GROUPING OF BACTERIA AND FISSION

When a bacterium multiplies by the process of transverse fission, two independent organisms arise which may or may not remain together for a time as a pair. There is a commonly observed tendency for actively multiplying bacteria to remain together in characteristic groupings, a feature of some usefulness in the identification of particular kinds of organisms. Bacteria may occur as single cells, pairs, short or long chains, irregular clusters, tetrads, cubical packets, or in palisades. Some of these arrangements are referred to by other names (fig. 3). The diversity existing in the groupings among bacteria is determined by differences in their growth properties and structure. Since the natural grouping of bacteria may be disrupted by physical or mechanical factors in their environment it is important to understand the causes of the grouping and to place a greater determinative value on these causal properties than on the actual observed grouping itself.

Grouping of bacteria is dependent on the geometric relation of successive planes of division and on the tendency for daughter cells to pull apart after division. If the successive planes of division are parallel, as they are in rod-shaped, spiral, and many coccoid shaped bacteria, the cells will occur singly, in pairs or in chains depending only on their tendency to pull apart after division. The palisade arrangement so characteristic of the diphtheroids is the result of a snapping, post-fission movement which swings the daughter cells in an arc from a pivot at the newly separated transverse wall and brings them to rest against one another with their long axes parallel.

In the case of some cocci the successive planes of division may occur in any direction. The organisms then are formed in irregularly arranged clusters. In other cocci the planes of division are perpendicular to each other and tetrads of cells result. In the case of the sarcina or cubical package arrangement, division is along three definitely oriented planes, the second being perpendicular to the first and the third division perpendicular to both the first and second division planes.

Due to mechanical agitation, convection currents in the environment, and Brownian movement, the expectation is that daughter cells should separate after fission. In the case of motile species this pulling apart should be further aided by the independent activity of flagella of the new organisms.

The occurrence of bacteria in characteristic groupings in spite of these

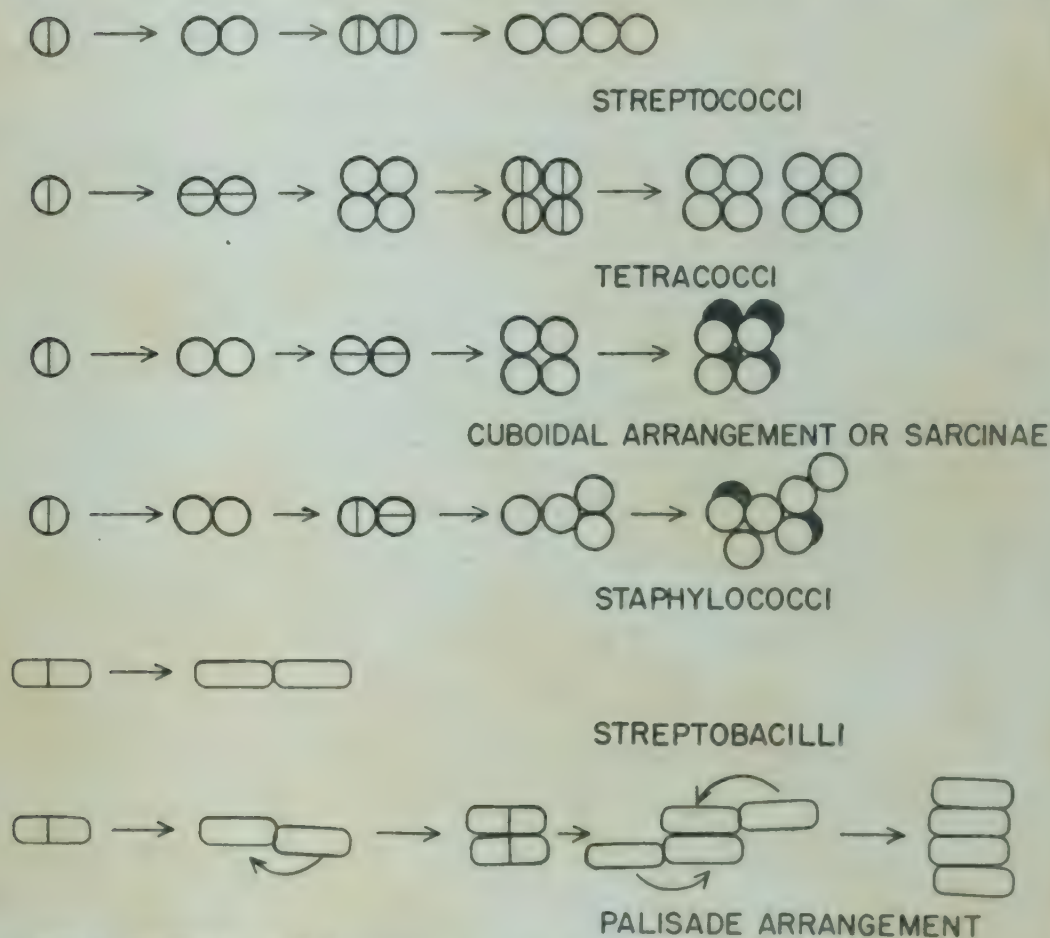


FIG. 3. Illustration of the various groupings assumed by bacteria.

forces favoring separation requires explanation. Fission has not often been described as a mere pinching off at the center of the bacterial body into two new organisms.<sup>1</sup> Rather a new transverse cell wall is laid down at the place where division occurs. Most often the growth of this new cell wall is described as beginning at the periphery of the cell, probably as a ring at the inner edge of the rigid cell wall and growing "centripetally" into the

<sup>1</sup> While models of the pinching off of droplets from larger drops of liquid under the forces of surface tension have provided amusement and instruction for the physical chemist, they have been of little value in explaining fission of biological origin.



interior. This process is probably preceded by the separation of the cytoplasm by the centripetal growth or invagination of the cytoplasmic membrane. From the descriptions in the literature it is not always clear whether or not the growth of new transverse cell walls is in two separated layers, one for each new daughter cell. If it grows as a single layer it could account for the lack of separation of bacilli after fission, so common in some species. On the other hand, it should be recognized that even for these species the new individuals do not remain together indefinitely. Therefore, even if the

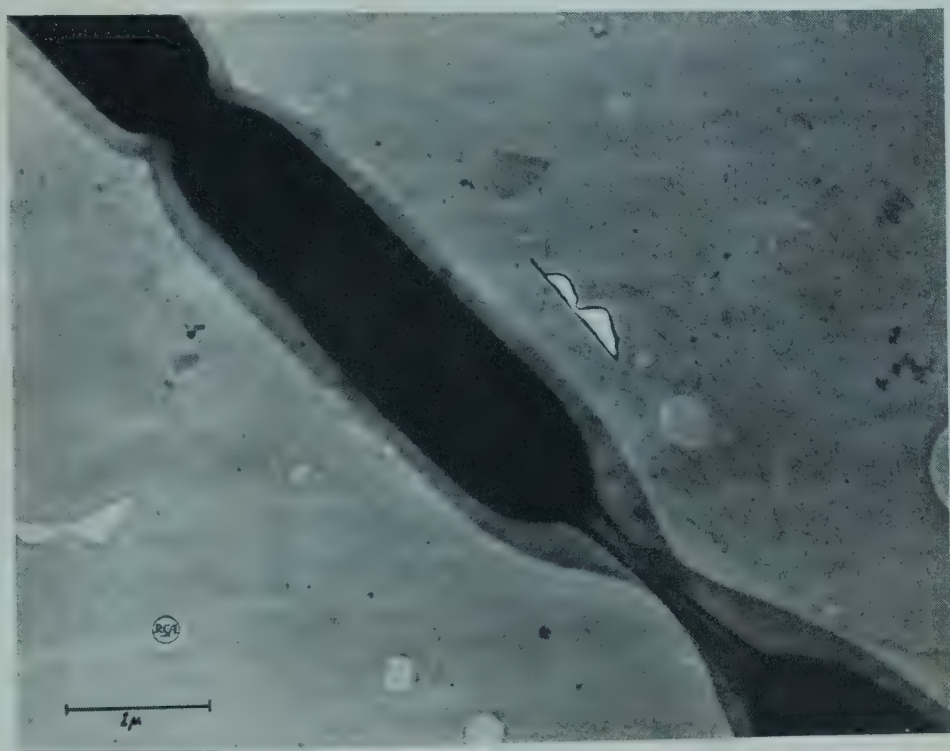


FIG. 4. A plasmodesmid in *Bacillus cereus*.

(From Johnson, 1944)

reason for the lack of separation were the original formation of a single-layered transverse septum, a mechanism must be postulated which could explain the eventual longitudinal splitting of the new cross wall. It may be that both possible situations occur. If so it would be desirable to determine in which species fission is accompanied by formation of single and double-layered transverse septa.

In some few cases the formation of the new septum may remain incomplete for a time. As a result a connecting strand of cytoplasm and/or cytoplasmic membrane between the two individuals prevents the separation. This connecting link is considered to be equivalent to the *plasmodesma* demonstrable between cells in many plant and animal tissues (fig. 4).

The surfaces of bacteria may be sticky, and when this is the case the basis for a lack of separation might be this property. In many organisms it can be shown that a highly viscous extracellular product is synthesized and accumulates at the cell surface. Adhesion of dividing bacteria coated with such substances might be expected.

### STRUCTURE OF BACTERIA

If the bacterial body were to be viewed in cross-section it would appear to be organized into four distinguishable layers, an outermost slime layer or capsule surrounding a rigid cell wall, and cytoplasm occupying the cavity delimited by the cell wall but separated from the cell wall by a cytoplasmic membrane. Structures corresponding to nuclei are probably present in the cytoplasm as well as vacuoles, droplets, and inclusions of varying size and composition. In addition, cells of motile species possess one or more flagella, long whip-like processes possibly cytoplasmic in origin and extending through pores in the rigid cell wall.

The structures composing organisms have been classified in various ways. A classification applicable to the bacteria and adapted chiefly from Arthur Meyer would be as follows:

#### I. Protoplast or living matter.

- a. Protoplasmatic structure: structures that do not arise *de novo*. Arise from a preexisting similar structure. Example: the nucleus.
- b. Alloplasmatic structure: structures that can be formed anew. Examples: cell wall, cytoplasmic membrane, flagella.

#### II. Ergastic or non-living structure: Examples: slime layer material or capsules, reserve food vacuoles, inclusions consisting of insoluble waste products excreted or precipitated within the confines of the cell.

The cytology of bacteria will be considered in greater detail in a later chapter.

### PHYSICAL CHARACTERISTICS OF BACTERIA

The characteristics of the bacterial organism include physical properties such as those of density, refractivity, and conductivity which have a role to play in bacteriological phenomena and which must be kept in mind when bacteria are observed or used for experimentation. There is a surprising scarcity of data on these properties and in general the work that can be quoted is not definitive. There have been so many improvements in biophysical instrumentation in the last two decades that a reinvestigation of the physical properties of large numbers of different kinds of bacteria would seem opportune. While the task of obtaining numerous data on the "fundamental physical constants" of bacteria might be a tedious one the reward

would lie in providing an authoritative body of readily available material for reference.

In speaking of the physical characteristics of bacteria it should be realized that two kinds of data are required, those on individual organisms and those on the mean quantity and spread for given populations of strains and species. While it may be interesting to know the properties of a given individual, it is generally more useful to know something about the statistical distribution of the property within a genetically related population.

#### SPECIFIC GRAVITY

The density or the specific gravity of the bacterial body is not much different from water. This is not surprising since the major component of protoplasm is water. The specific gravity of bacteria has been quoted as low as 1.07 and as high as 1.19. Obviously this property depends on the proportion of substances present in the cell having a specific gravity greater than water. Such substances would be proteins (1.5), carbohydrates (1.4–1.6), nucleic acids (2.0), and mineral salts ( $\sim 2.5$ ). The presence of lipid material with a specific gravity of less than one would tend to reduce the density. Since the chemical composition of bacteria varies under many different circumstances, the specific gravity of bacteria can only be characteristic for fixed sets of conditions of growth.

The weight of the living bacterial organism has been determined statistically by dividing the weight of a mass of cells by the number of cells. The order of magnitude is  $1 \times 10^{-9}$  to  $1 \times 10^{-10}$  milligrams.

The vertical distribution of bacteria in liquid and gaseous environments is in part dependent upon their density which determines their resistance to sedimentation. A convenient means of rapidly separating a population of bacteria from such environments is centrifugation, hence it is useful for the bacteriologist to be thoroughly familiar with the physics of sedimentation. For our purposes the sedimentation of bacteria may be regarded as merely an example of the settling of solid particles in a fluid medium and taking place at a rate expressed by Stokes' law.

Two forces act on the falling particle: (1) the frictional force resisting the fall of this particle and (2) the gravitational force acting on the particle. The net result is a maximum velocity for the falling object achieved when the two opposing forces become equal. For a spherical particle falling at constant velocity the frictional force is taken equal to

$$f_1 = 6\pi\eta r dx/dt \quad (1)$$

where  $\eta$  is the viscosity of the fluid,  $r$  the radius of the particle, and  $dx/dt$  the velocity of fall. The gravitational force causing the fall is

$$f_2 = mg \quad (2)$$



where  $m$  is the "effective mass" of the particle, and  $g$  is the acceleration due to gravity. This expression may be modified to

$$f_2 = 4/3\pi r^3(d_p - d_f)g \quad (3)$$

where  $d_p$  is the density of the particle,  $d_f$  is the density of the fluid, and  $4/3\pi r^3(d_p - d_f)$  is the "effective mass" of the particle. Since at constant velocity  $f_1 = f_2$ , then

$$6\pi\eta r \frac{dx}{dt} = 4/3\pi r^3(d_p - d_f)g \quad (4)$$

which is Stokes' law.

Various applications are immediately apparent since solution for any one of the several factors is possible, and in practice the various quantities may be evaluated experimentally. For example, the velocity of fall of a bacterial cell may be predicted from knowledge of its size and density and from the properties of the fluid:

$$\frac{dx}{dt} = \frac{2r^2(d_p - d_f)g}{9\eta} \quad (5)$$

In a similar way the size of the cell, viscosity of the medium, etc. can be obtained from knowledge of the appropriate combinations of pertinent factors.

For the strict application of Stokes' law in the form developed here, several requirements must be met. In the first place deviations occur when the particle is not spherical although corrections may be made in some cases if the actual shape of the particle is known. Furthermore, the law applies only to particles in a given size range depending on the nature of both the particle and the fluid and on the strength of the "gravitational" field acting on the particle. In general, the falling particle must be at least an order of magnitude larger than the molecules of the fluid medium and **larger than the mean free path<sup>2</sup> of the fluid molecules.**

If centrifugal fields are employed in lieu of the earth's gravitational field, Stokes' law still may be employed by substituting the appropriate acceleration for  $g$ . Since  $g$  may now become very large, small particles (still subject to the above shape and size restrictions) may be investigated. The well known use of the ultracentrifuge depends upon this possibility.

In theory a particle large with respect to the fluid molecules should fall through that fluid even though that particle is actually quite small. However, in practice the rate of fall of quite small particles is so low as to become submerged in other effects, particularly Brownian movement. Only

<sup>2</sup> The mean free path is the average distance a molecule travels between its collisions with neighboring molecules.

when the velocity of fall is greatly increased by high centrifugal fields can measurements be made in the range of colloidal particles. Actually, painstaking and prolonged experiments indicate that even the very small sucrose molecules will definitely sediment at a very low rate in an aqueous solution subject only to ordinary gravitational acceleration. But aside from the verification of Stokes' law, practical use cannot be made of studies in such systems.

Finally one very important restriction on the use of Stokes' law must be emphasized. Any turbulence in the fluid will affect or even completely offset expected sedimentation. Applied directly it will be seen that convection currents in natural bodies of air or water will strongly oppose the sedimentation of bacteria. While such mixing is obviously important in sedimentation studies, unfortunately its evaluation is quite difficult. Therefore, the procedure adopted in general laboratory practice involves elimination of turbulence, obviously an impossibility when one is concerned with large natural systems. In general, therefore, Stokes' law would predict that bacteria could occur only at the bottoms of air and water masses. Convection currents, then, are responsible for the occurrence of bacteria in suspension at high levels. The net result of the operation of both processes would be a greater average density of bacteria near the bottom of the system with large point to point variations throughout the fluid.

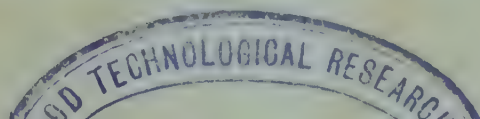
#### REFRACTIVE INDEX

There are available only a few data on the refractive index of bacteria and none on the variations within a clone. In general the refractive index is slightly greater than for water. The values recorded are usually less than 1.45 but do range up to 1.55. Bacterial spores lie at the top of this range. Strictly considered it is inaccurate to speak of the refractive index of the bacterial body since the refractivity varies across the individual structures of the organism.

#### CONDUCTANCE

The electrical conductance of suspensions and tissues has been regarded as an important tool in the study of membrane phenomena. However, significant conclusions have not thus far been obtained. It seems clear that the interiors of cells show a high conductance (conductance is the reciprocal of resistance, hence the resistance is low). Since the cells as a whole have a low conductance, the cell membrane is believed, by default, to be the element of low conductance (high resistance).

At the present time the data on the action of electrolytes on suspended cells seems rather ambiguous, and it is even uncertain that the presence of salts alters the conductance of bacterial cells. It has been hoped that



the permeability of cell membranes to electrolytes might be elucidated by conductance studies. So far, clear-cut effects of salts on the conductivity are not found nor is there even a definitely known difference between the conductances of living and dead bacteria.

### CHEMICAL COMPOSITION

The chemical composition of bacteria resembles that of other organisms in the nature of the elements and compounds present. The one exception is the absence of sterols in bacteria other than *Azotobacter chroococcum*.

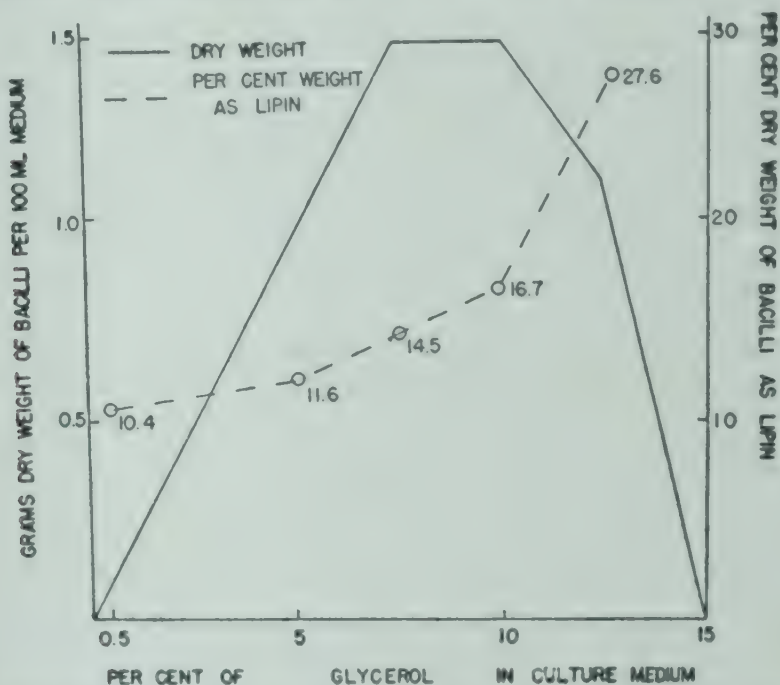


FIG. 5. Relation of the lipid content of the tubercle bacillus to the glycerol content of the culture medium.

(Adapted from Long and Finner, 1927)

This type of compound is otherwise universally distributed in living organisms. The elementary composition of bacteria reveals oxygen, hydrogen, carbon, nitrogen, and phosphorus as the major constituents. These elements together with lesser quantities of potassium, magnesium, calcium, sodium, sulfur, and chlorine constitute the bulk of the elementary composition. There have also been identified in still smaller or trace quantities iron, aluminum, manganese, copper, boron, and more infrequently a variety of other elements. Reports of the ash content have varied from one to 14 per cent.

The major constituent of bacteria is water, reported as 75 to 85 per cent



of the total. The organic matter is composed of protein 40 to 80 per cent, carbohydrate 10 to 30 per cent, and lipid 1 to 30 per cent. Lipid may be present as "visible," refracting droplets, as "masked" finely dispersed material, or in combination with proteins. Nucleoprotein may compose up to 80 per cent of the organic matter in certain species. Nucleic acid is both of the pentose and desoxypentose types and has been reported to occur from as low as 5 per cent of the dry substance to as high as 30 per cent. Vitamins, pigments, and other organic compounds in bacteria are present

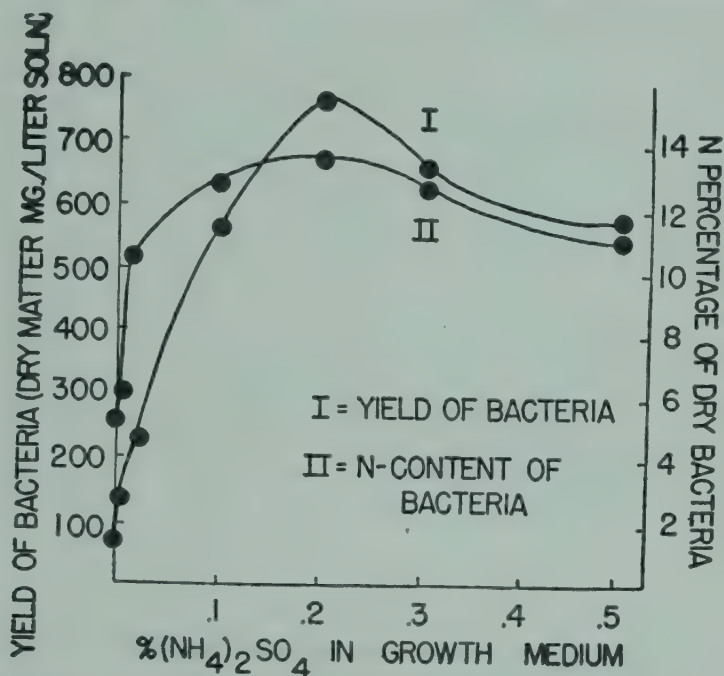


FIG. 6. Relation of the yield of bacteria and nitrogen content of the organisms to the concentration of assimilable nitrogen in the growth medium.

(From Virtanen and DeLey, 1948)

as quantitatively minor constituents. All of the known amino acids have been identified in bacteria except diiodotyrosine and thyroxine. Compilations of data on chemical compositions are to be found in Buchanan and Fulmer (1928) and Porter (1946).

The acid-fast bacteria (genus *Mycobacterium*) differ from other bacteria and from living forms in general by the unique nature of their lipids. The fats of the mycobacteria are fatty acid esters of trehalose rather than esters of glycerol. Their phosphatides do not contain choline and ethanolamine, but they do include unique liquid saturated fatty acids. Specific and new carbohydrates, high molecular weight alcohols, and hydroxy acids have also been isolated from these bacteria.

Within limits the chemical composition of bacteria varies with the nature of the organisms. Thus while most bacteria contain more protein than carbohydrate, the rhizobia or root-nodule bacteria have been reported to contain relatively more carbohydrate. Also the chemical and physical characteristics of the growth environment modify chemical composition. Growth in media containing high concentrations of mineral salts can dramatically increase the ash content of bacteria, and media rich in carbohydrate may result in a greater accumulation of lipid. Figure 5 shows how the total dry weight and the lipid content of the human tuberculosis bacillus vary with the concentration of an oxidizable substrate in the medium. The total nitrogen content of bacteria varies with the concentration of assimilable nitrogen in the medium (fig. 6). More interesting is the fact that the nature of the enzymes synthesized by the bacterial cell is influenced by the nitrogen content of the medium (see Chapter 9). The qualitative dependence of the chemical composition of bacteria on the nature of the medium is also indicated by the synthesis of certain slime layer polysaccharides only when specific carbohydrates are present in the medium. For example, *Streptococcus salivarius* produces abundant slime layer polysaccharide only in the presence of sucrose or raffinose. The production of particular enzymes, the adaptive enzymes, in response to the presence of specific substrate in the medium is also illustrative of the dependence of the composition of bacteria on the nature of the growth medium.

### THE ROLE OF WATER

Among the enormous number of compounds making up any living organism, water is undoubtedly the most abundant and is certainly to be regarded as one of the common denominators of life as we know it. As such it deserves some special attention. The wide distribution of water on the earth is so well recognized that one occasionally forgets the vital role it plays and overlooks important phenomena based at least in part on the presence of water.

Let us consider for a moment the far-reaching consequences of the density of water. Since this material makes up a large proportion of living matter, it will make an important contribution to the average density of that matter. In other words, living things will vary in their densities about the density of water and will tend to be only a little lighter or heavier than water. It may be seen that with only slight density differences to overcome many forms in the animal kingdom have developed mechanisms for moving vertically through water, ranging in character from methods of swimming to plunging from air and floating upward once submerged.

Turning from the general contribution of the density of water, let us consider the effect of change in density on freezing. In general most sub-

stances contract on freezing, and the solid thus formed will settle through the remaining liquid. Obviously if ice were heavier than water and settled out as it formed, all large bodies of water would be frozen solid except perhaps for shallow upper liquid layers which might exist during a brief period of the warmest weather. One might expect comparatively severe weather in the coastal areas of the world, and desert conditions with higher temperatures inland due to low atmospheric humidity due in turn to the low vapor pressure of the cold or frozen oceans. Higher marine life could probably not exist other than in a few shallow bodies of water unfrozen the year round. Only simple cold resistant organisms as bacteria, primitive plants and the like could grow in any open water available during the warmer season. The indirect effects on terrestrial life would be great when one considers possible climatic conditions and our present concept of the origin of terrestrial species from marine ancestors. In fact one might doubt that life based on water could ever have begun were it not for the expansion of water on freezing and the existence of a point of maximum density above the freezing point.

Similar contemplations on the relationship between water and living things result from the consideration of other properties of water, and in many instances these properties appear to be unusual. For example, water may be looked upon as a member of certain chemical families and compared in various ways with the members of these families. Let us illustrate with two such groups, one organic and one inorganic:

INORGANIC			ORGANIC		
Substance	Formula weight	Boiling point	Substance	Formula weight	Boiling point
H <sub>2</sub> O	18	100°C.	H <sub>2</sub> O	18	100°C.
H <sub>2</sub> S	34.1	-59.6	CH <sub>3</sub> OH	32	64.7
H <sub>2</sub> Se	81.2	-42	C <sub>2</sub> H <sub>5</sub> OH	46.1	78.4
H <sub>2</sub> Te	129.6	-1.8	<i>n</i> -C <sub>3</sub> H <sub>7</sub> OH	60.1	97.8
			<i>n</i> -C <sub>4</sub> H <sub>9</sub> OH	74.1	117
			<i>n</i> -C <sub>12</sub> H <sub>25</sub> OH	186.3	255.9

Ordinarily when the boiling points of closely related compounds are compared they are found to correlate with the molecular weights. In the above compilation water seems to be exceptional and to boil at a higher temperature than is anticipated by such reasoning. By similar comparisons anomalous behavior is noted in many if not most of the properties of water. Among those worthy of mention are the density maximum, high surface tension, almost uniquely high specific heat, high freezing and boiling points, high latent heats of fusion and vaporization.



All these various anomalies have been generally interpreted as arising from peculiarities in the structure of liquid water. The consensus of present opinion is that water in the liquid state contains aggregates of the basic structure represented by  $\text{H}_2\text{O}$ . Unfortunately the extent and duration of such associations cannot be determined simply and directly. Hence interpretations and even the data themselves have been vigorously questioned, and it is not yet possible to definitely picture the architecture of water. There is little doubt that some sort of aggregation, association, or polymerization of  $\text{H}_2\text{O}$  does exist to form the naturally occurring water substance and that the process and its results are responsible for the unusual properties of water. In other words the average molecular weight of water at any given time is larger than the formula weight for  $\text{H}_2\text{O}$  although the exact value is unknown and probably represents the weight of a statistical structure subject to rapid formation and destruction.

Turning more specifically to bacteria it is obvious that much of the foregoing may be directly applied. However, there are certain other factors which are of great importance in the functioning of such small organisms as bacteria, and these are problems which may be conveniently approached using the relatively simplified systems available to bacteriology.

Some concepts of the structure of water have suggested the existence of definite polymers diminishing in size with increasing temperature. This hypothesis has been less generally extended to include the assumption that the interconversion of these polymers might be slow as the temperature changes. In other words, it was thought by some investigators that freshly boiled or condensed water when cooled to a given temperature would be less polymerized than water held at that same temperature for a long period. If such is the case might not the vital processes of living systems be affected by such differences?

Growth experiments using *Escherichia coli* and *Euglena gracilis* in media containing freshly condensed water on the one hand and "aged" water or freshly melted water on the other purportedly showed greater growth in the latter case. It seems probable, however, that the growth data reported arose from some other variation such as lack of control of the amount of dissolved gases. It will be immediately apparent that carbon dioxide (known to stimulate growth in certain cases) is very low in concentration in freshly condensed or boiled water. The investigations showing such growth differences did not control the carbon dioxide content and are to be criticized on this basis. In fact subsequent experiments failed to verify the growth differences previously reported. Finally a large variety of data involving measurements of vapor pressure, magnetic susceptibility, infrared absorption, and density lead one to the conclusion that equilibrium in the molec-

ular association is reached very rapidly, too rapidly for measurement by any available method including growth rate measurements.

#### LIFE AND HEAVY WATER

The effect of heavy water on living matter has received widespread attention in the past because reactions involving hydrogen transfer might conceivably be slowed enough by the heavy hydrogen atoms to block metabolic processes. Single doses of heavy water administered to higher organisms have little apparent result. However, in such cases the relative amount of "ordinary" water within the animal is large, and the heavy water would be rapidly and extensively diluted. When higher animals were continually fed heavy water ( $D_2O$ ) instead of ordinary water, serious effects occurred such as liver damage, necrosis in the spleen, and eventually death. Such animals behaved as though they were extremely thirsty. In these experiments it is not certain that the destruction is limited to chemical alterations by heavy water since there might be a considerable osmotic gradient causing tissue injury.

On the other hand, a number of isolated enzymes whose action involves hydrogen transfer definitely show reduced activity when deuterium replaces hydrogen. It is conceivable, therefore, that biological processes are so slowed or thrown out of balance that death results. Many of the smaller organisms die or suspend their normal processes in high concentrations of deuterium oxide. In these cases osmotic injury is more easily avoided by gradually increasing the amount of  $D_2O$ . Lower concentrations, perhaps up to 30 per cent, permit apparently complete activity of yeast, bacteria, and various simple animal organisms although some reduction in activity may take place and result in an increased life span as in *Planaria*. Quite low concentrations of deuterium in the water, down to water essentially free of the heavy isotope, seem to have little effect on organisms. Some reports have indicated that slight excesses of deuterium stimulate growth as might a small quantity of particular toxic substances. However, the experiments were not well controlled with respect to pH and other factors and may not be valid since the phenomenon has not been observed in other cases by other investigators.

#### WATER AND METABOLISM

In solvent power water is unequalled in its ability to dissolve the majority of the components of cells. Dispersion of most proteins, carbohydrates etc. is difficult in any other solvent, and only in water can one conceive of the transport of metabolites, waste products, proteins and even cells as we know them. Normally growing, metabolizing cells of all kinds



are bathed in an aqueous phase, for if the organism does not live in water or a rather moist medium, then the water is supplied internally as in the higher plants and animals.

In addition to acting as an inert medium for vital systems water also has other fundamental roles. Photosynthesis and many metabolic reactions require water as a reactant. On the other hand, it is formed in many metabolic processes and represents a final step in the oxidation of compounds containing hydrogen and the acquisition of useful energy by the organism. While the water content of living material is high and is usually in continual and rapid transfer across cellular membranes, the exchange does not lead to unlimited dilution of the cellular components under ordinary conditions. The regulation has been correlated with that of certain gels which appear to imbibe water to a limit and then cease.

General osmotic control appears to be lost by a cell in a strong salt solution when it is broken open by sudden, extensive dilution (a process called osmotic shock). In such cases the soluble contents of the cell are dispersed throughout the solution and osmotic phenomena no longer occur.

#### THE BOUND WATER CONCEPT

The process of water loss for cells and tissues exhibits some unusual aspects. For example, the succulent stems of a cactus placed in a desiccator over sulfuric acid lost only 10 per cent of the total water in a six months' period and sprouted when returned to a moist atmosphere. Observations of this type have been generally attributed to imbibition forces which are regarded as being analogous to or the same as the forces responsible for the hydration of ions and molecules. It should be pointed out that the presence of soluble materials in solution, waxy coverings like those on cacti, the nature of the cell wall, and so forth will greatly affect the loss of water and may be the major factors in preventing water loss.

The fact remains, however, that the complete removal of water from many proteins is quite slow and requires exposure to atmospheres of low humidity and low total pressure to expedite evaporation. The reasons for such tenacity are not clear but have been widely assumed to resemble the forces of hydration which sometimes resist very strongly the removal of water of hydration. In the case of proteins, other large molecules, and protoplasm in general, firmly fixed water has been known by the term *bound water* and much use has been made of the concept. It seems to be implicit in general attitudes toward bound water that this material is rather different from water substance (free water) and not at all, or at most quite slowly, in equilibrium with water in bulk.

Operating on the hypothesis that bound water would freeze at abnormally low temperatures many cryoscopic data have been taken in efforts to



estimate such properties as resistance to freezing, heating, drying, and aging. In practice protein solutions, starch pastes, gels, and tissues begin to freeze at temperatures somewhat below  $0^{\circ}\text{C}$ ., the exact temperature depending upon the rate of cooling, dissolved materials, and crystal nuclei formation. Crystallization of ice continues and eventually slows but may be extended by lowering of the temperature. Many investigators have claimed, however, that cooling at reduced temperatures for many days will crystallize only a portion of the total water and that the unfrozen residue is "bound water".

The proportion of such bound water is then correlated with the resistance of the organism to various adverse conditions. For example, the well known resistance of bacterial spores has sometimes been attributed to low total water content. However, when investigators failed to find significant differences between the water contents of spores and the respective vegetative cells, the bound water was then compared by cryoscopic methods in the two forms of several bacteria. A conclusion was reached that the proportion of bound water was higher in the spore than in the vegetative organism and that the ratio of free to bound water paralleled the thermal death rate at high temperature.

On the other hand, several serious objections to the validity of the concept of bound water have been raised. Materials which crystallize as solid hydrates are well known and contain water in the crystal lattice, so one would consider it a firmly held or bound part of the molecule. Yet on passing into solution the rigidity of the crystal is at once lost, and the situation becomes so greatly altered as to become uncertain. It is clear that ions (and probably neutral molecules) are hydrated in solution, but in this case the system is considered to be a dynamic one with a rapid exchange of the water molecules grouped about an ion. In other words, the orientation of the water is statistical only in relation to the ions and varies markedly from point to point and time to time.

Another major obstacle in applying the bound water concept is encountered in the analysis for total water in biological systems. It is quite evident that such determinations are at best only estimates since the absolute value depends greatly on the method used, even to the slightest details. Part of the difficulty in many procedures is due to the actual removal of the water which causes separation of the solutes from solution. Many of these solutes deposit as films around droplets and effectively reduce the diffusion of water from such droplets into the surrounding system. Furthermore, no accurate knowledge exists of the stability of the large molecules to drying. Obviously decomposition of proteins does occur during drying with loss of water of composition before all of the free water can be removed from the interior of the material. Hence the determination of total water in biological

material becomes an arbitrary procedure and places bound water to total water comparisons on an unsound basis because of uncertainty in the total water.

Returning to a discussion of the bound portion of the water, one might anticipate that a number of methods should be available for its measurement. In general, these will depend upon measurement of (a) the thermodynamic activity of the water in the system (b) or the amount of ice formed on freezing. Since many of the methods are related and a rather large number have been used, only selected procedures will be considered here.

A logical approach and one used very widely is the determination of freezing point lowering caused by the addition of an extra, inert solute. Basically the method requires knowledge of the total water, the freezing point of the system, and the freezing point after addition of a known quantity of a solute such as sucrose. If the amount of water available for solution of the sucrose is unaffected by the proteins, etc. present then the lowering of freezing point produced by the added solute will be exactly that expected from the quantity of water and solute present. If, on the other hand, bound water exists in the system and is unavailable for solution of the sucrose, then the amount of "free water" will be less than the total water and the freezing point lowering will be unexpectedly large by an amount related to the fraction of bound water.

Several investigators employing this cryoscopic method on protein solutions, bacteria, and tissues have observed freezing point depressions which led them to propose bound water in amounts of a few per cent to a considerable fraction of the total water. It should be pointed out that the data tend to be badly scattered when repetitions are made. This lack of accuracy in the measurement may be due to any combination of several causes. In more concentrated systems where the relative effect becomes greatest, very serious supercooling may occur even with ice seeding. Likewise the high viscosity of such solutions retards crystal growth by coating the solid surface with a solute film formed when the pure solvent separates as a solid phase and leaves behind local zones of relatively much higher solute concentration. Also because of the viscosity of the concentrated systems, heat transfer during freezing is retarded. These various difficulties are magnified in the presence of the solute (usually sucrose) since the addition of large quantities of solute increases the viscosity still more. In general they are all reflections of lack of equilibrium which may completely negate the interpretation of the results. As a matter of fact lyophilic colloids (the type important here) are thought never to be in thermodynamic equilibrium and there may then be fundamental theoretical difficulties in assessing any measurement where attainment of equilibrium is necessary. Finally the cryoscopic method assumes that the solute has the same thermodynamic



activity in the colloidal system that it does in simple aqueous solution, a state of affairs that is definitely not always obeyed, and in general, cannot be demonstrated.

Another method on which much work has been done is the determination of the total amount of ice formed when the sample is frozen as compared to the amount of ice expected from the total water content. In general this process is a matter of ascertaining the volume change on freezing the colloidal system and comparing it with the change in volume for water equal to the total water content of the system. In this dilatometric method somewhat erratic results in the same range as those of the cryoscopic method are obtained. Commonly the system is held at  $-20^{\circ}\text{C}$ . and the unfrozen water remaining is considered to be different from bulk water and is, therefore, called bound. However, another explanation for failure to freeze exists. Equilibrium may not be attained. Certainly the extent of freezing increases on extended holding at the low temperature, and a further temperature decrease freezes even more water.

Two other types of systems that may be analogous are the high stability of liquid water drops in atmospheric clouds at quite low temperatures and 4 per cent water in toluene emulsions in which 19 per cent of the total water does not freeze after 21 hours at  $-36.7^{\circ}\text{C}$ . It is felt that freezing equilibrium is not achieved in such cases because crystallization nuclei are limited to those water droplets which do freeze. Other droplets having no nuclei of their own and not containing the crystalline material, remain liquid because equilibrium is not established owing to lack of contacts between the droplets. As the water separates in a tissue the solute concentration rises until precipitation occurs and isolates water which then will not freeze no matter what the temperature because it cannot be seeded. It should be pointed out in this connection that the spontaneous formation of nuclei becomes quite slow at low temperatures, and freezing ceases because of their absence.

Several other methods related to the two discussed above have indicated bound water, but in general the difficulties and criticisms are much the same. In other cases experiments indicate that "bound water" if it exists at all, does not differ from the "free water" of the system. In other words rapid exchange of "water of hydration" with "water of solution" is indicated. This is shown by:

- 1) When a sucrose-protein-water solution is ultrafiltered the ratio of sucrose to water in the hydrated protein residue is the same as it is in the protein free filtrate to within a very small amount which may be considered experimental error.

- 2) Vapor pressure lowering caused by the addition of an inert solute to a protein-water system is exactly that expected from the amounts of solute



and total water present. Undoubtedly equilibrium needs consideration here, but if involved at all it would seem to point in the direction away from tightly fixed and extensively modified or "bound" water.

Since the present authors feel that the concept of bound water in its original sense is not justified by the facts, the problem of resistance of cells and spores to heat or freezing cannot be resolved by the application of the bound water concept.

### METHODS FOR STUDY OF CHEMICAL COMPOSITION

Study of chemical composition may be undertaken with the view of establishing the nature of structures or the distribution of particular materials within the cell. *Microincineration* is a technique for the study of the location and distribution of ash within the cell. In this procedure the organisms are ashed directly on a glass slide. After incineration the slide is examined under the microscope without displacing the ash residue. The physical properties of the ash can be studied with the various types of microscopes. Often carbon may be identified directly. Qualitative analysis for the elements may be done using micro-color or precipitation reactions. While microincineration of bacteria has not been used extensively, work reported with *Spirillum rubrum* suggests the adaptability of the technique to bacteria.

Staining procedures have been used most often for study of the chemistry of bacterial structure. More recently the methods of histochemistry have been successfully applied to the problem of the distribution of enzymes within the bacterial body.

Analysis for the chemical components of bacteria is not a simple matter. Methods of gross chemical analysis have been used and are particularly satisfactory for indicating elementary composition. Spectrochemical analysis is also reliable for this purpose and for detecting trace elements. However, there are numerous obstacles in attempting to determine the nature of the compounds present in bacteria. While it is possible to determine the percentage composition in terms of the general nature of compounds present, i.e., proteins, fats, etc., it is not often possible to determine with accuracy particular compounds by methods assuring the collection of the natural constituent as it occurs in the cell. In order to get at the natural intracellular constituents a most critical need is for methods of disruption of bacterial structure without chemical change. Solvent extraction, while useful for some purposes, certainly does not obviate the need for methods for breaking up bacteria.

Possible techniques for rupturing the bacterial body are numerous. Actually these methods have been more often employed to isolate enzymes in

studies with cell free preparations than for the investigation of chemical composition. Various methods are listed and discussed briefly:

1. Lysis, dissolution of cells. *Bacteriolysis* is the lysis of bacteria. Lytic phenomena are open to the objection that they involve chemical change both as a primary cause of lysis and subsequent to the lysis. The disruption of structure may well result in enzymes attacking cellular constituents from which they are otherwise isolated in the intact cell.
  - A. Autolysis. Many bacteria undergo a spontaneous lysis under particular cultural conditions. This phenomenon may lead to the presence of high molecular weight materials of bacterial origin in culture filtrates.
  - B. Induced lysis. Any lysis resulting from the action of a foreign agent or from the addition of an extraneous substance to bacterial suspensions. Actually there has been little work done on the possibilities of using lysis of this type for the isolation of cellular constituents.
    - ✓ a. Bacteriophage. An ultramicroscopic agent or virus which specifically infects particular strains or species of bacteria and generally causes an explosive dissolution of the infected bacterial body.
    - b. Lysozyme, a carbohydrase lysing some bacterial species. Widely distributed in animal secretions and present in some vegetable tissues.
    - c. Complement. In the presence of complement and specific antibody bacteria may be lysed. Gram negative species seem to be the most susceptible.
    - d. Amino acids. It has been shown that bacteria may be lysed by addition of certain amino acids to broth cultures. Glycine seems particularly effective.
    - e. Surface active agents. Chemical substances that are powerful surface tension reducing agents may cause lysis. The synthetic detergents are an ever expanding source of this type of potentially useful lytic agent.
2. Change in permeability. Any substance which when added to bacterial cultures causes a change in permeability is a potentially useful tool for separating constituents from the cell. For example, some detergents while not lytic are known to cause a leakage of nitrogen compounds from the bacterial body.
3. Mechanical rupture, any physical method of fracturing. These methods have been preferred to those already mentioned. Presumably their advantage and wide acceptance lies in the inherent probability that these methods, more than others, should permit the isolation of intact cellular constituents. Nonetheless it cannot be assumed that chemical change

does not accompany the mechanical disruption of the cell. For one thing enzymes normally in restricted zones of the cell are free to diffuse after the cellular structure is disturbed. Also, some of these methods are accompanied by a significant and deleterious rise in temperature. The latter objection is usually met by working at controlled low temperatures. It would also seem desirable in principle to use these methods on dry organisms, but this possibility is still one open to future investigation.

- A. Crushing between glass slides. Small as they are, bacteria fixed to a glass slide can be crushed by simply applying pressure and twisting a superimposed glass slide or cover slip with the thumb. Obviously the small quantities of bacteria that can be crushed in this way do not permit use of this method except when specific and sensitive microchemical tests are available for direct application under the microscope.
- B. Hydraulic pressure. Cellular liquid can be squeezed out of a cake of bacteria by the application of from 400 to 500 atmospheres pressure with a press.
- C. Sudden release of pressure. By exposure to pressures of inert gases of the order of 3000 to 6000 pounds per square inch and sudden release of the pressure it is possible to break bacteria open. An advantage of this technique is that the rupture of the organism is accompanied by a precipitous drop in temperature.
- D. Freezing and thawing. Alternate freezing and thawing will result in the mechanical disintegration of bacteria. This very useful method is not ideal since some proteins can be denatured by this procedure.
- E. Supersonic waves. Supersonic apparatus has been designed for use with bacteria. The method is a practical one particularly where large quantities of material are not needed. The amount of material that can be exposed to supersonic waves at one time is limited in volume.
- F. Agitation. By shaking bacteria with inert particles of the proper size it is possible to rupture them. The particles employed need not have sharp edges, indeed smooth-surfaced glass beads of a 60-80 sieve size have been found to be very effective. Unfortunately, thick suspensions of organisms cannot be handled with this procedure. Bacterial spores, generally the most difficult bacterial objects to destroy by mechanical means, are successfully ruptured by agitation with smooth, inert particles of the proper size.
- G. Grinding. Various methods of grinding in mills have been described. The Booth-Green wet crushing mill is an especially efficient instrument. Ball milling can also be used by which either wet or dry cells may be ground.



Grinding with an abrasive such as powdered pyrex glass or carborundum (mesh 600RA) is practical. Usually a thick paste of bacteria with two to ten parts by weight of abrasive is used. For rapid grinding of a large percentage of the organisms it is necessary to use a large excess of abrasive. This relationship is disadvantageous since it adds to the problem of separating the product from the abrasive and increases the surface area of abrasive available for possible adsorption of cellular materials. Grinding with an abrasive can be done by hand with a mortar and pestle as well as with a mill. There are also a number of devices described in the literature for hand or mechanical grinding by movement of a mixture of abrasive and bacteria in a thin layer between opposing glass surfaces.

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## CHAPTER IV

# Microscopy

### THE ORDINARY COMPOUND LIGHT MICROSCOPE

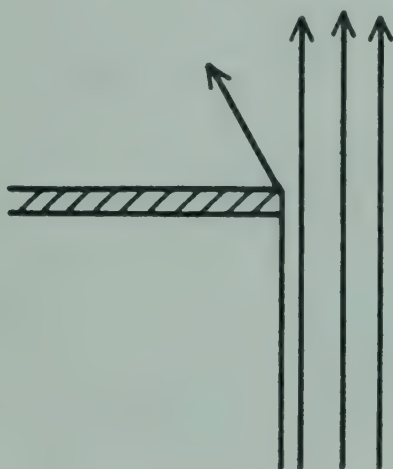
The light microscope has been designed and is used for the observation of small objects and their fine structure. Hence its primary function is one of resolution or visual separation of closely spaced objects. Magnification achieved with the instrument then assumes the secondary role of increasing the apparent sizes of the resolved objects to the point of visibility by the eye.

In a consideration of resolving power certain limitations of the human eye must be taken into account. Most important, the light that forms the image in the eye falls on the ends of sensory receptors of finite size and spacing, and the light received by any single receptor appears as a point. Therefore, if the rays from two closely spaced points fall on a single receptor, only one point will be observed; in other words, the two objects will not be resolved. It is generally believed that the eye sees two distinct objects only if they are separated by a *visual angle* of one minute or more. The *visual angle* is the angle made by the border rays of light at the nodal point or optical center of the eye when they cross on their path to the retina.

It follows that the closer the system is to the eye the greater will be the visual angle that the object subtends, and thus resolution will increase with decreasing distance of the object. Unfortunately the eye has a practical limit in accommodating for distance, and the average person is able to resolve adjacent objects best at a distance of about 250 mm. The compound microscope is actually used as a device for effectively bringing the eye exceedingly close to the object, the degree to which it is able to do so being limited by the properties of its component parts. The optics of the instruments of standard design adjust for accommodation for distance by presenting a virtual image at a distance of 250 mm. from the eye. Thus paradoxically the microscope effectively brings the observer to within a few millimeters of the object, and yet one is actually viewing an image of the object at the optimum distance of 250 mm. Actually the extent to which the microscope can fulfill its function is remarkably near the theoretical limit imposed by the physical properties of materials available and the wavelengths of light used.

With the compound microscope the object is supported and illuminated.

An enlarged image of the object is formed by the objective, and this image is further enlarged by the eyepiece. Without going extensively into the structural features and laws of optics let us consider first the function and effective utilization of the objective. Light striking a small object is partly transmitted directly upward and partly diffracted<sup>1</sup> at an angle (see fig. 7). When a beam is formed by passing light through a slit or past an edge, the central portion of the light beam is transmitted without alteration in the direction of the individual rays. However, the rays of light at the edges of the beam are scattered away from the central rays, the relative extent of



## LIGHT BEAM

FIG. 7. Illustrating the diffraction of light at the edge of an object. Light at the edge is deflected by an amount depending upon the size of the object.

scattering depending upon the wavelength of the light and the shape and size of the beam. Obviously the smaller the beam the greater its circumference relative to its cross section, therefore, the greater will be the proportion of light scattered and the greater the angle of the scattered (diffracted) light with respect to the undiffracted rays.

According to one theory of resolution the microscope objective collects both normal and diffracted rays (fig. 8) and from them subsequently forms the image. The angle of diffraction depends upon the dimensions of the object under observation and is greater with respect to the normal when the spacing of the components of the object becomes less. If two objects are so close as to diffract light at  $90^\circ$  then obviously the objective cannot re-

<sup>1</sup> The term diffraction is used to describe the scattering of light at the edges of a pencil of light.

ceive the diffracted rays, and resolution of the objects cannot be achieved, for at least part of the diffracted rays must be collected if resolution is to occur. Stated in other terms the *angular aperture* (the degree of obliquity of the light collected or the *visual angle*) of the microscope must be great enough to include part of the diffracted radiation. It has been found in

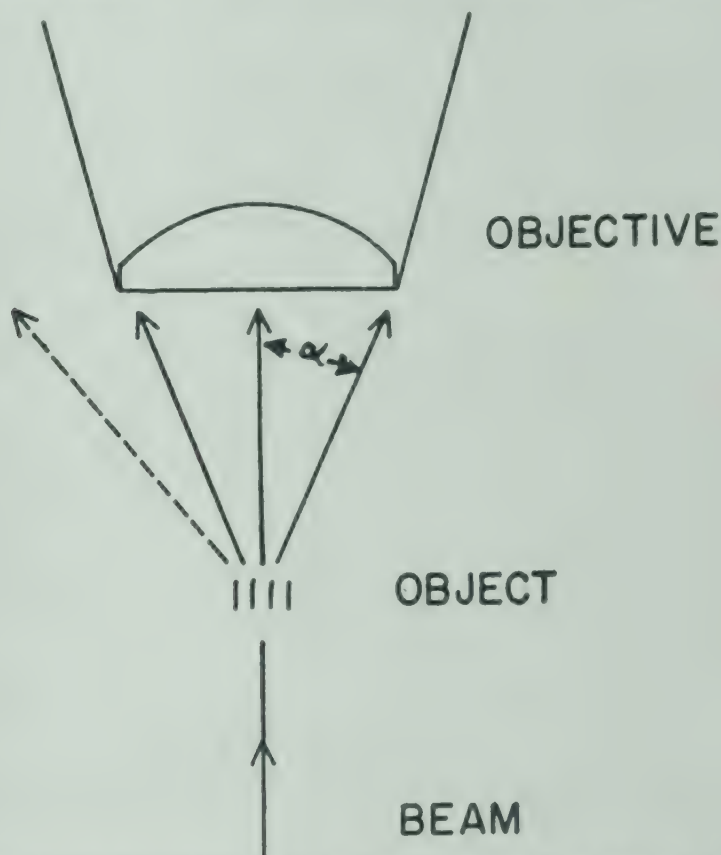


FIG. 8. The angular aperture of an objective. The object diffracts rays at an angle  $\alpha$  with respect to the normal ray.  $2\alpha$  represents the maximum divergence of rays that the objective lens can collect and is called the angular aperture. If the angle of diffraction exceeds half the angular aperture (as indicated by the dotted ray), the diffracted rays will not be collected, and the spacing in the object will not be resolved.

practice that the *working aperture*, usually called the *numerical aperture*, depends upon both the angular aperture or capacity to capture oblique rays and the refractive index of the medium.

$$\text{Numerical aperture} = (\text{refractive index}) \times \left( \sin \frac{\text{angular aperture}}{2} \right) \quad (1)$$

where the angular aperture equals twice the angle of the received ray having the greatest diffraction or obliquity with respect to the normal. The importance of large numerical aperture is emphasized in the following ex-



pression for the limiting resolution:

$$d = \frac{0.6\lambda}{\text{numerical aperture}} \quad (2)$$

where  $d$  is the smallest distance by which objects may be separated and still appear as separate objects and  $\lambda$  is the wavelength in vacuo of the illuminating radiation.

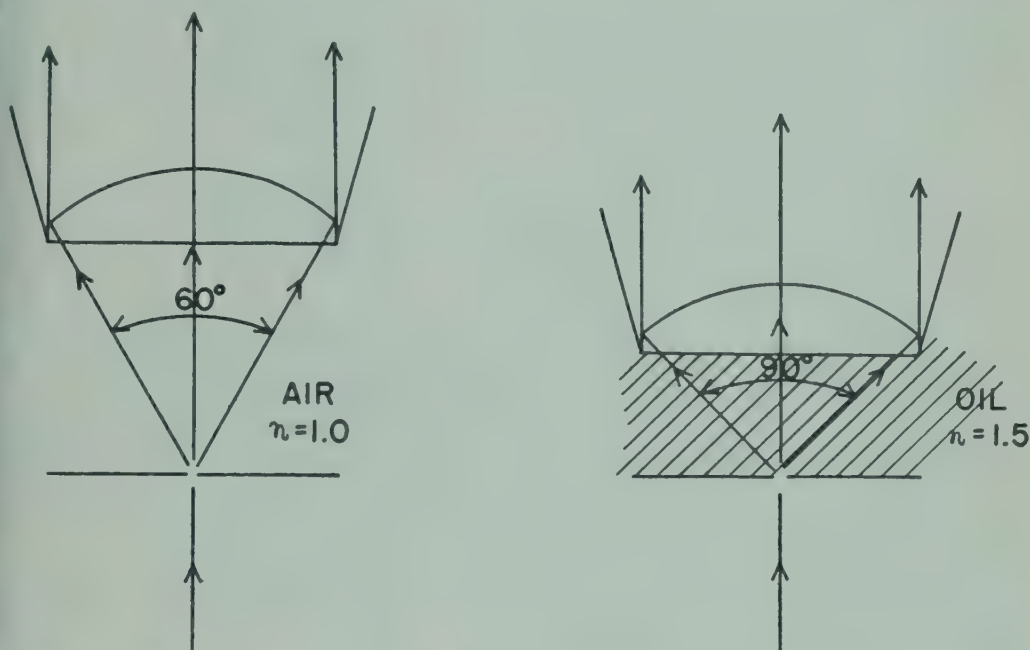


FIG. 9. Showing the effect of oil immersion on the angular aperture of objectives during observation of unmounted objects. As is illustrated the focal length of the objective is much shorter in oil than in air. Thus the oil immersion objective is brought much closer to the object and consequently collects more divergent rays than does a dry objective of the same area. In other words, an immersion medium of high refractive index increases the angular aperture and the resolving power of an objective.

One would expect from the above considerations that low power objectives having a relatively long focal length would need to be of large diameter in order to collect the most oblique rays and thus to possess high resolution. Manufacture of such objectives becomes difficult and expensive and they are inconveniently large, so the result is that the usual long focal length objective has low resolving power. However, when the focal length is short the outer lens surface is very near the object, permitting the use of lenses that are relatively small in diameter but still able to collect rays at very wide angles.

Three devices are available for obtaining high numerical aperture. The

first is the grinding of large diameter, short focal length lenses. Secondly, since the refractive index of the medium is involved this index may be considerably increased with important increases in resolution (fig. 9). It will be recalled that the angular rays emerging from a medium of high refractive index into one of low index will be bent further from the normal and if the angle is great enough will be completely reflected. This difficulty arises especially when mounted objects are observed with dry objectives so that light from the object must pass through air before being received by the objective. Fine or closely spaced structure is not resolved under these conditions because the diffracted rays are bent beyond the edge of the objective. When oils of high refractive index are used such rays are collected by the objective and resolution occurs.

The third device employed involves the use of oblique illumination. Since numerical aperture depends upon the angle between the normal ray and the most diffracted ray collected by the objective, it is possible to increase greatly the useful angle by tilting the normal ray away from the principal axis of the microscope. If, however, a single inclined beam were used the illumination of the object would be unsymmetrical and unsatisfactory. Hence it is customary to employ a system of lenses called a condenser which brings the illuminating light to focus on the object as a cone of oblique rays thus providing uniform illumination (fig. 10). In this way the condenser becomes an important adjunct of the objective in all microscopes of high resolving power, and since its numerical aperture controls the obliqueness of the illuminating rays it also influences the effective numerical aperture of the objective itself.

The microscope eyepiece serves the function of enlarging the image formed by the objective until the fine structure resolved by that objective is large enough to be resolved by the observer's eye or on a photographic plate. Since the eyepiece merely makes resolution observable but does not increase it, excessive magnification by the ocular serves no useful purpose.

In order to employ the maximum available resolution of an immersion objective of a given short focal length several conditions must be met. Beginning first with the condenser it is found necessary to use a condenser of numerical aperture at least as great as that of the objective, otherwise the maximum useful obliqueness is not achieved and the greatest possible angle of the diffracted rays collected is not maintained. If the numerical aperture is greater than that of the objective no increase in resolution occurs and much contrast actually is lost as compared to the situation when both numerical apertures are equal. Therefore, to avoid changing condensers when the objective is changed, an iris diaphragm is provided to reduce the numerical aperture of the condenser. The correct setting may be achieved

by viewing the back of the objective and adjusting the diaphragm until the back lens is just filled with light. Naturally the condenser must be centered with respect to the objective to yield uniform illumination of the object. Since highest numerical apertures are not achieved in systems possessing low refractive index, air is replaced by oil of high refractive in-

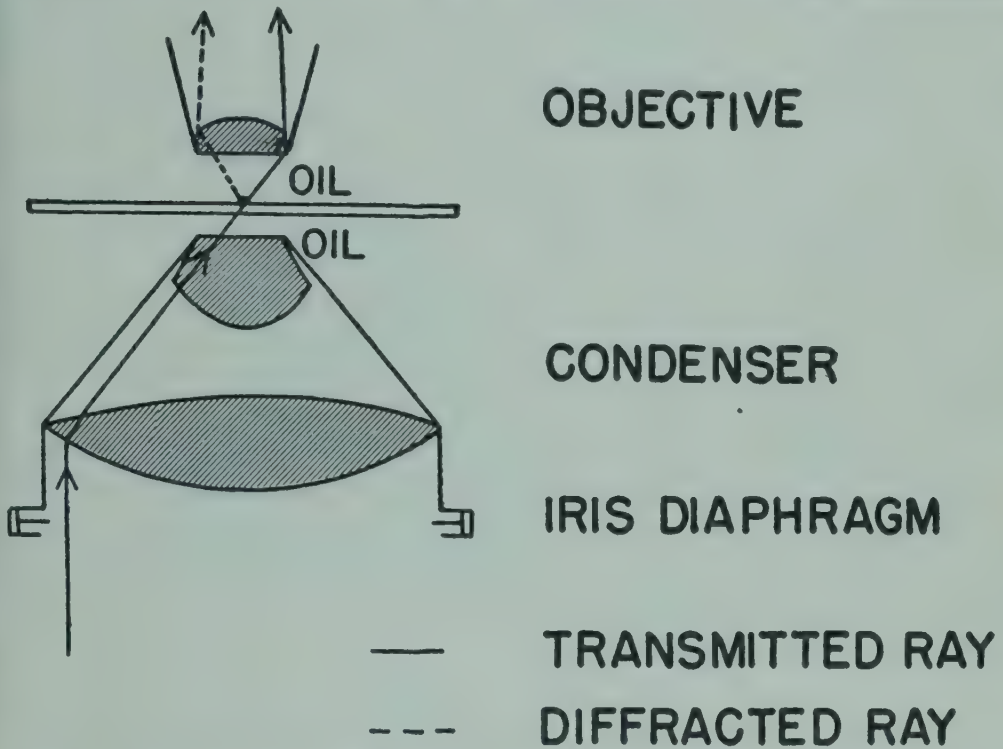


FIG. 10. An Abbe condenser. Note how use of the oblique illumination permits collection of widely diffracted rays and effectively doubles the angular aperture. The numerical aperture of both the condenser and the objective should be the same for best results. When low aperture objectives are used the aperture of the condenser is decreased by adjusting the diaphragm.

dex between the condenser and the material supporting the object (usually a glass slide) as well as between the object and the immersion objective.

For mounted objects the mounting medium should possess as high a refractive index as the other components of the system. High index oil should be used between the mount (or object if unmounted) and the objective to avoid loss by reflection of the rays needed for resolution. It should be emphasized that the numerical aperture of the objective is limited by the lowest refractive index present in the system including the immersion oil, slide, cover glass, oil between condenser and slide, mounting medium if used, and the glass from which the objective and condenser are made. Any



attempted improvement of resolution via refractive index changes must therefore always involve an alteration of the component having the lowest index.

The resolution in the objective image then needs only to be developed by the combined magnification of the objective and the eyepiece. The physiology of the eye demands only a total magnification of about 350 times the numerical aperture of the objective. However, owing to eye strain that may ensue from prolonged observations near the limit of resolution, this factor should be increased to the neighborhood of 700. Sometimes for the mere counting of objects it may be desirable to increase magnification still further although caution must be used in observations of structure in all such cases. In general, eyepieces capable of a magnification of about ten diameters are powerful enough for work with the most commonly used objectives.

Lastly, resolution increases as the illuminating wavelengths decrease and considerable advantage may be taken of this fact, especially in photography, where ultraviolet light may be used to greatly improve resolution. It is customary to select special light sources that are rich in short wavelengths or to employ filters that remove the undesired longer wavelengths when doing either visual or photographic work. Ultraviolet microscopes have been employed where extreme resolution was required, but at wavelengths below about 3200 Å. quartz lenses are required, an expensive arrangement unjustified except for special circumstances.

### DARK FIELD ILLUMINATION

With small transparent objects the contrast between the objects and the field is low, and interpretation of the image formed by an ordinary microscope becomes difficult. However, contrast in the image is greatly increased if the field can be kept dark while only the object appears to be illuminated. This effect is achieved with both dark field illumination and the polarizing microscope.

In principle, microscopes adapted for dark field illumination differ from standard models only in the type of condenser employed, the condenser supplying light whose *minimum aperture is greater than the maximum aperture of the objective*. This relationship keeps any direct rays of light from entering the objective, and only rays reflected or diffracted by the objects in the field are collected to form the image. As a result the object appears to be self-luminous in a dark field. See Figure 11 for a diagrammatic representation of this scheme.

Since the minimum aperture of the condenser is only slightly greater than the aperture of the objective, the condenser must be accurately centered with respect to the optic axis of the objective, otherwise some rays of light will enter at one edge of the objective and a dark field will not be

maintained. Also the condenser must be sharply focused on the object, or the object will not be illuminated since it will lie above or below the zone of light at the focal point of the condenser. Inasmuch as only a small portion of the light from the source is used for illuminating the object and none of the direct rays passing through the field are collected, dark field microscopy requires an intense source of light.

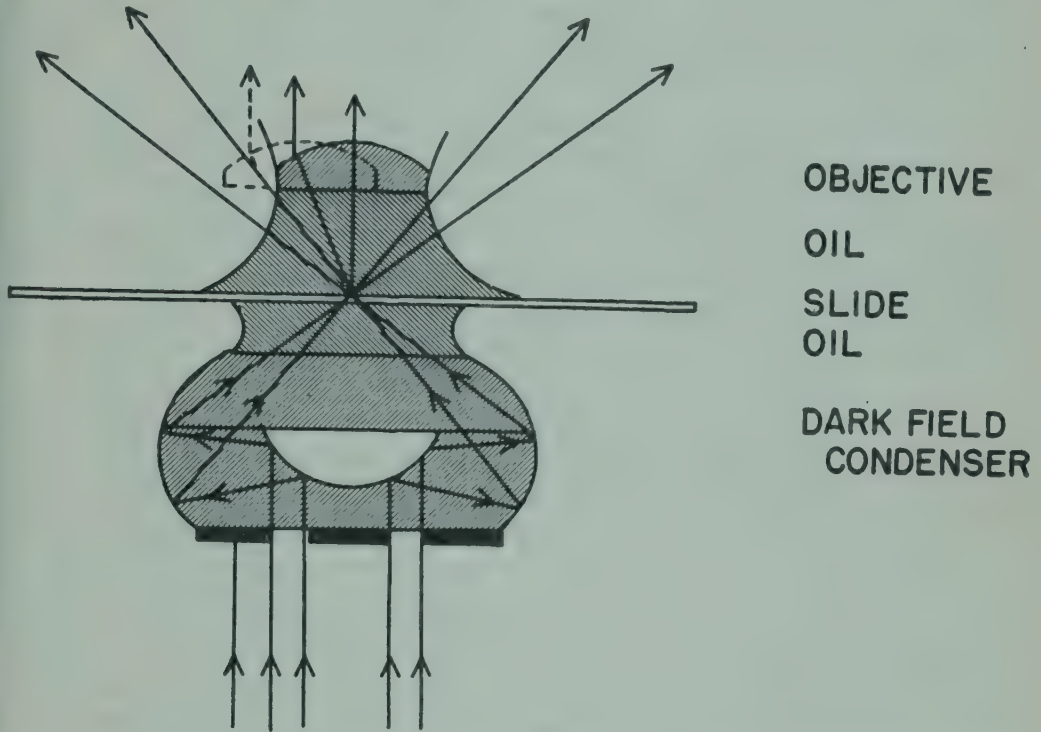


FIG. 11. Dark field illumination. Light enters the condenser through an annular opening and by internal reflections a divergent ring of light is brought to focus on the object. Immersion oil may not be necessary depending on the apertures involved, but ordinarily a high aperture is needed in the condenser and oil must be used to prevent total reflection. The dark areas at the base of the condenser represent opaque plates or metallic films. The diagram is of course not in scale, particularly in the vertical dimension.

Dark field illumination considerably reduces the depth of focus of an objective as compared to ordinary bright field illumination. This change must be borne in mind to avoid errors in the interpretation of structure. On the other hand, the two methods of illumination result in comparable resolution for a given objective. It will be apparent that the numerical aperture of objectives used in dark field work cannot be as high as the highest apertures used in bright field studies because of the geometrical limitations imposed by the need for very high apertures in the dark field condensers.

The good resolution available accompanies excellent contrast in dark field microscopy. In bright field observations if structures are resolved they still may not be clearly apparent due to lack of contrast, while with the same objective and dark field illumination the structures will be much more evident to the observer because of the inherent high contrast. It is this fact which makes dark field illumination a valuable supplementary method to bright field microscopy and has led to its use in research on the morphology of small organisms and the routine characterization of some bacteria.

In one aspect care must be taken. With a very intense source of illumination the diffracted intensity from the object is so great that a diffraction ring surrounding the object may appear in the dark field. Thus objects tend to appear of greater dimensions than they actually are, and this illuminated area may be falsely interpreted as an outer envelope or structure. Slowly closing an iris diaphragm placed *behind* the objective causes this spurious pattern to shift away from the object, a shift that would not occur if a real structure were involved. Another test for the presence of a diffraction ring involves reduction of the intensity of illumination at which the ring will disappear although the real object still remains clearly visible.

Internal structures may be emphasized, when desired, by mounting cells in Canada balsam or oil having a refractive index close to that of the exterior of the object under observation. Mounted in such a way the outer structures merge into the field and other portions having different refractive indexes can be seen more readily. Some work has also been done with selected wavelengths for the illumination since in many cases different cells or structures may appear to be of different colors, and differentiation is thus possible. Such work may be assisted by staining of the organisms prior to observation.

### THE POLARIZING MICROSCOPE

This instrument has been widely employed in chemistry, mineralogy, metallurgy, and to a lesser extent in other fields, where it has proven to be of great value in the identification and study of materials. Its usefulness depends upon certain characteristic optical properties of crystalline particles.

Typical crystals fall into one or the other of two categories known as *isotropic* and *anisotropic*. The former group is quite small consisting of only a few simple inorganic salts like sodium chloride, all of which belong to the cubic system of crystals. The thirty or so known isotropic crystals exhibit properties which are independent of the orientation of the crystal or of the direction of measurement. On the other hand the members of the



anisotropic class total hundreds of thousands of crystalline substances, and all of these materials possess properties whose quantitative measure does depend upon their orientation when the measurements are made.

Using the property of refractive index, let us illustrate. It will be recalled that the refractive index of a material is a measure of the reduction in the velocity of light as it passes through that material. Stated quantitatively the refractive index of a sample of matter is the ratio of the velocity of light in vacuo (or in air which is very slightly less) to the velocity of light in that matter. Expressed in this way the refractive index is always greater than unity and is found to depend on the nature of the material in question. Among the gases, liquids, and isotropic crystals the magnitude of the refractive index is not a function of the direction at which light enters the material. For anisotropic materials, however, the velocity of a ray of light does vary as it passes through the material in different directions. Hence not only is the magnitude of the refractive index a characteristic property of the material but also of the particular way in which it is affected by the orientation of the material.

Anisotropic bodies ordinarily are rigid solids of regular structure and as such possess three dimensions. In one large group there are three different refractive indexes corresponding to the three dimensions. In another group of substances only two different refractive indexes are observed; these materials may be thought of as having the same refractive index in two of the three possible directions. Both of these types are subdivisions of the anisotropic class. Extending this approach, the isotropic materials may be regarded as possessing three refractive indexes corresponding to the three dimensions but with all three being equal.

Since refractive index measurements may be easily made with considerable accuracy and since the number of possible different combinations is exceedingly great when three are involved, a knowledge of the refractive indexes would be of great value in the identification of materials, especially crystals. The necessary measurements require the passage of light through the material in a single direction at a time. This illumination can be accomplished by using plane polarized light and altering the relative position of the illuminated object as required.

As the name implies the polarizing microscope is equipped to polarize the light illuminating the object. It does so by means of Polaroid discs or Nicol prisms.<sup>2</sup> Using such light, measurements may be made if the object

<sup>2</sup> Optical materials sold under the trade name *Polaroid* contain uniformly oriented crystals of optically active organic compounds embedded in a film such as cellulose acetate. The very small crystals and their orientation confer on the system the property of filtering out all the incident light except that vibrating in one plane.

can be variously oriented by rotating the stage. Frequently the desired positions for refractive index measurement lie at odd angles with respect to the crystal faces, and a so-called universal stage is then required which can be used to rotate the crystal in any plane by any desired amount. Unfortunately at present, this latter stage arrangement is useful only for particles considerably larger than bacteria.

However, the polarizing microscope by means of a second polarizing element (called an *analyzer*) placed above the object will reveal existing anisotropic character in bodies ranging down toward the size of bacteria. The optics involved and the limitations will not be discussed. Suffice it to say that the existence of oriented structures can be demonstrated in the larger cells and in these cases inclusions might be correctly characterized as solid or liquid in nature. It is most unfortunate that present optical systems require a relatively large total size for the object. Were it otherwise, much information concerning the structure and composition of bacteria would become available without the risk of chemical changes leading to artifacts, always a possibility in the common staining procedures.

### PHASE CONTRAST MICROSCOPY

In addition to the use of dark field and polarizing microscopes, contrast in the image may be improved by means of the phase contrast microscope. Contrast in the image of the ordinary microscope results from differential absorption, reflection and diffraction. However, light traversing two objects will emerge out of phase if one object is thicker or has a slightly greater refractive index than the other. When the rays from two such differing objects are brought together and are out of phase, they interact to produce *interference* (darkness) or *reinforcement* (brightness). In phase contrast microscopy this is deliberately accomplished by separately treating the rays coming directly from the source through the medium and the other rays scattered by structures in the object.

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Transmitted light, therefore, is confined to one plane of vibration and by definition this is plane polarized light.

Ordinary light passing through many kinds of clear crystals is separated into two components vibrating at angles to each other and each component emerges plane polarized. In the case of large clear crystals of calcium carbonate (Iceland spar, calcite) the crystal may be cut and cemented back together in such a way that the light vibrating in a particular one of the two planes strikes the cemented surface at an angle greater than the critical angle and is reflected. This light emerges from the side of the crystal and is absorbed in the mount. The remaining light vibrating in a different plane passes through the cemented joint and on through the crystal face opposite the face of entry. To summarize, a beam of light is resolved into two component polarized beams one of which is reflected and absorbed and the other emerges from the Nicol prism as plane polarized light.



The instrument employed is an ordinary light microscope which has been provided with an annular diaphragm located at the lower focal plane of the condenser and a diffraction plate located in the objective system. Light comes from the condenser as a hollow cone of parallel rays and is collected by the objective. It is then brought to focus on the annular ring of the diffraction plate and is partly absorbed, retarded or unaltered depending upon the material composing the ring. Finally the light is collected to form the image (fig. 12).

With an object in the field its structures reflect and diffract light, so that each scattering structure acts as a light source. The diffracted rays (dotted line in the diagram) fall over the entire diffraction plate and therefore are mainly affected by the principal plate areas, the annular ring portion being of relatively small area. The diffracted rays are also brought into the plane of the image, and phase differences are there changed into intensity differences as the result of interference phenomena. If the direct rays from the source and the diffracted rays are in phase, a bright image of the object structure is obtained. If they are out of phase a black area results, and intermediate conditions yield a series of grays (fig. 13).

The diffraction plates consist of glass discs variously coated, the ring area differing from the remainder of the surface. The plate must be so located in the objective system and possess an annular ring of such dimensions that all the rays direct from the source fall on the ring, yet the ring must be as small as possible to avoid collecting a large proportion of the relatively weak diffracted intensity. Light through this ring may be retarded by a layer of dielectric material, partly absorbed by a thin metal film, retarded and partly absorbed, or passed unchanged through the bare glass plate. The remaining plate area affecting the diffracted light may be coated in any one of the same ways and the various possible combinations will yield a number of different types of diffraction plates. Furthermore, the extents of retardation and absorption may be independently and widely varied. In practice only a small number of plates (of the great variety that are possible) are actually used.

Phase contrast microscopy is especially useful in biology because the contrast of biological structures in their natural environment is often so low as to be invisible. As a matter of fact bacteria may be located in tissue sections when they cannot be seen by ordinary bright field methods or even by dark field observation since in the latter case reflection and diffraction by the entire section may be relatively great and nearly the same as for the bacteria. Phase contrast microscopy in addition to its major use in biology has begun to contribute to the study of other materials as textile fibers, thin sections of minerals, and the like.

In general, the resolution of phase contrast objectives corresponds to



that of bright field objectives of the same aperture although at the present time the available objectives are somewhat lower in aperture for phase contrast work. It will be apparent that for each objective differing in numerical

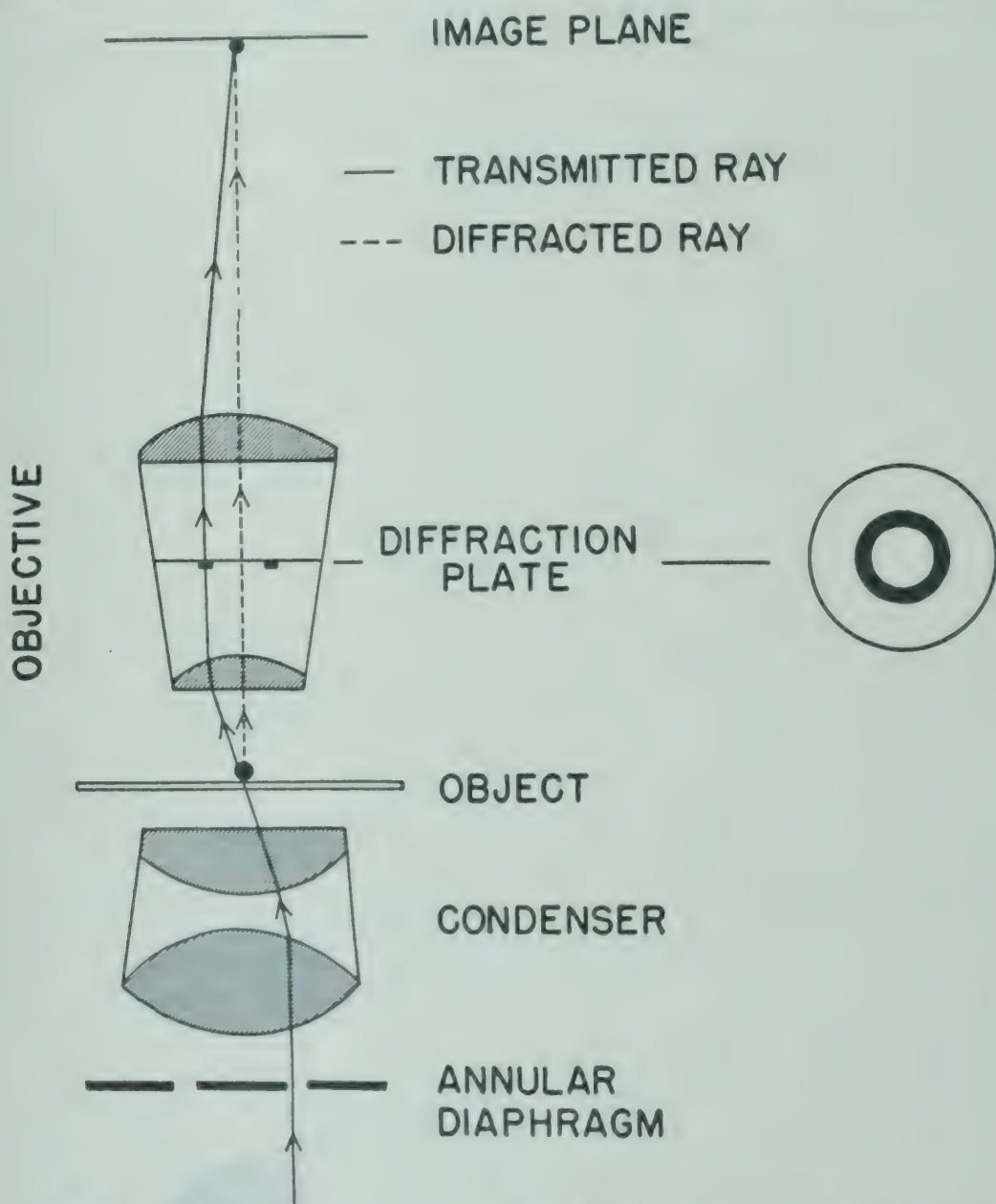
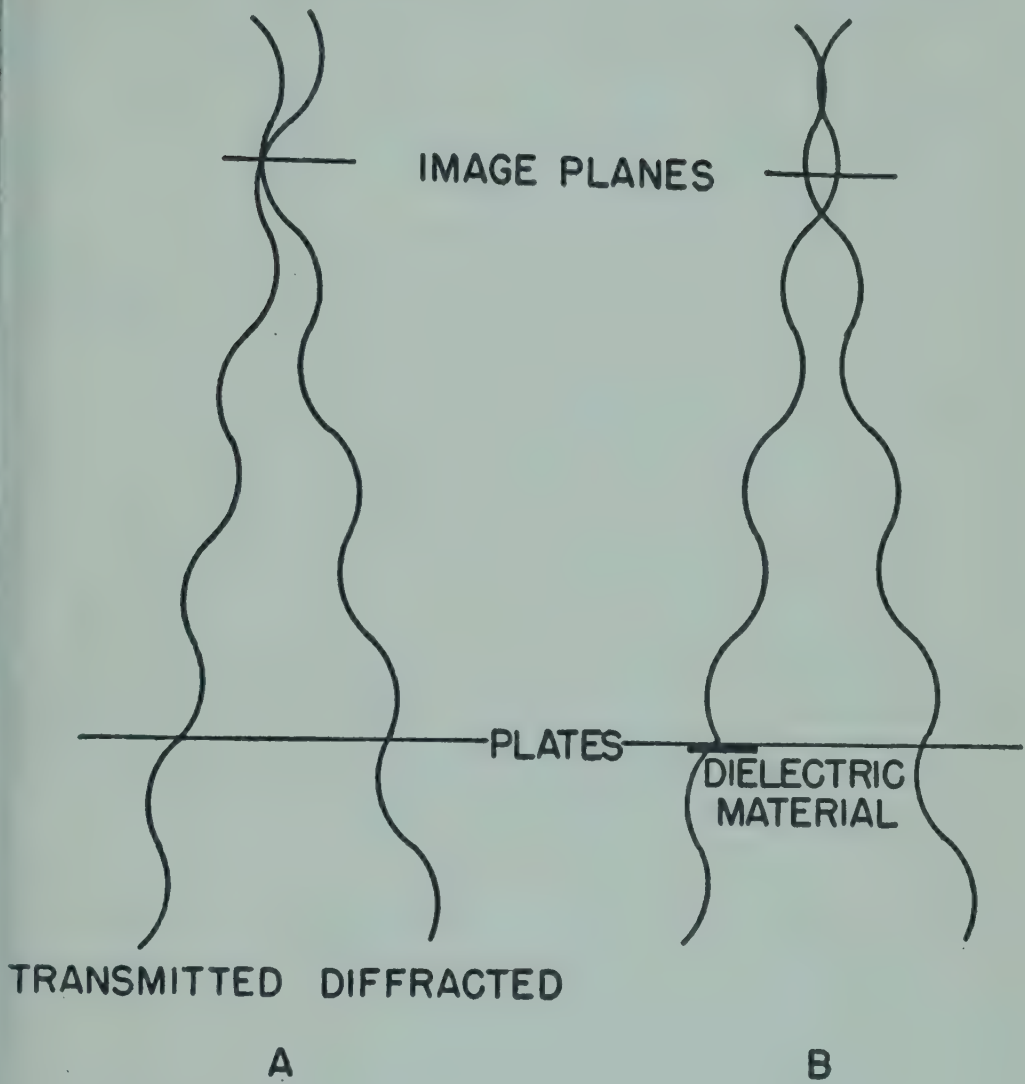


FIG. 12. A phase contrast microscope. The light source is focused on the annular diaphragm which is opaque except for the ring. The solid lines indicate the paths of sample rays direct from the source. Dotted lines represent diffracted rays originating at the object structures illuminated by the source. The diffraction plate is also shown in cross-section with a ring corresponding to that in the annular diaphragm. Phase interference (or reinforcement) occurs in the image plane and produces the desired contrast in the intensities of the image.

REINFORCEMENT

INTERFERENCE



## RAY FROM OBJECT THROUGH OBJECTIVE

FIG. 13. Illustrating the principle of light interference as involved in the phase microscope. In microscopy rays both transmitted and diffracted by a small object are brought to focus in the image plane. If the rays arrive in phase they reinforce each other producing a bright image. On the other hand, if they arrive out of phase they oppose each other and a dark area results. In the diagram the rays are shown as waves which possess characteristic amplitudes (intensities). The amplitudes at the image plane are added algebraically to determine the light intensity of the image. In A both amplitudes have the same sign, so the combined intensity is greater than that of either ray separately. In B the amplitudes are equal in magnitude but opposite in sign, so the combined intensity is zero. By proper adjustment of the relative phases all variations from complete interference to complete reinforcement are possible.

aperture a corresponding diffraction plate will be required, and the location of the plate with respect to the objective will likewise depend upon the focal length of that objective. The selection of the type of diffraction plate giving the greatest contrast with a given specimen is still empirical although widespread experience now permits a certain amount of generalization and manufacturers can make valid recommendations.

### THE LEPTOSCOPE

It has been found that the thickness of a film deposited on a standard surface can be determined by measuring the intensity of light reflected from such a film. Since the intensity of the incident illumination and the refractive indexes of the superimposed film, the standard supporting surface (usually a glass), and the external medium (usually air) are involved, they must be known. The leptoscope is a device designed to compare the intensities reflected from an unknown surface film and from a standard film of known thickness. Essentially it is a comparison microscope especially designed to provide an equal intensity of vertical illumination for both the known and unknown objects.

The standard films may be a series of monomolecular barium stearate films applied step-wise to the surface of a slide made of high refractive index glass. Since reflection differences depend upon the difference between the refractive index of the film and that of the glass, the glass used should have the highest possible refractive index. Usually a compromise will be required since very high index glasses are also very soft and are easily scratched and etched. In practice, the standard films are a series of stages of double film layers providing a sort of micro-stairway of film thickness with each stage representing a thickness standard. The intensity of light reflected from the object is bracketed between the two nearest stages of the step film. The thickness is then calculated using an interpolated thickness of standard film equivalent to the unknown and correcting for any refractive index difference of the unknown film relative to that of the standard film (both relative to the glass). Obviously if the thickness is known the refractive index may be estimated and the leptoscope may be used to demonstrate variations in either the thickness or the refractive index of unknown materials. It has been successfully used, for instance, in the study of erythrocyte envelopes or ghosts.

Owing to the smaller size of bacteria, only limited study can be made with the leptoscope especially since the bacterial body is spherical, ellipsoidal, or cylindrical resulting in a continuous variation in thickness across any dimension of the organism. If there were large irregular discontinuities in the cell surface either in contour or refractive index, the leptoscope should



readily reveal them since very small relative differences will yield contrasting reflection intensities. In general the conventional methods of microscopic examination and staining will reveal any discontinuity in bacteria that might be shown by the leptoscope, although in theory the reflection principle employed with the leptoscope should be valuable in demonstrating structural zones in cell surfaces. In practice, however, the bacterium is so small that even the gross structure is at the limit of resolution of the present leptoscope, and fine structure in the surface of the cell therefore could not be differentiated. Possibly future instruments may extend the range of usefulness to objects as small as bacteria.

### THE ELECTRON MICROSCOPE

The ordinary light microscope has a resolution of about  $0.2 \mu$  which is close to the theoretical limit of resolution, and further improvements are most likely to involve the fidelity of the image or contrast in the image rather than resolution. Fortunately, another instrument has become available that is capable of much greater resolution, namely, the electron microscope.

In this microscope, waves of electrons transmitted through a vacuum are used for illumination instead of light waves. Much shorter wavelengths thus become available for increasing resolution. The wavelength  $\lambda$  in the electron beam depends upon the accelerating voltage:

$$\lambda = \frac{150}{V} \quad (3)$$

where  $V$  is the accelerating potential. In the usual cases with potentials of 50,000 volts  $\lambda = 0.055 \text{ \AA}$ , a value very much less than is available in the useful electromagnetic range. At the present time the magnetic lenses used to refract the electron beam are free of aberrations only at low numerical apertures near 0.01. The expected limiting resolution  $d$  then becomes

$$d = \frac{0.6\lambda}{\text{N.A.}} = \frac{(0.6)(0.055)}{0.01} = 3.3 \text{ \AA} \quad (4)$$

a value that, though not yet achieved, is closely approached by present day instruments.

Several additional factors in connection with the electron microscope deserve mention insofar as they supplement or extend data obtained with the ordinary light microscope. First, pictures of very high quality are obtained due both to the use of short wavelength and of small aperture with the net result of a reduction in eye strain. The small aperture used permits a great depth of focus relative to that of a powerful light microscope and

allows one to "see" the entire vertical dimension of small objects rather than merely a thin section. Since in electron microscopy, fixing, staining, etc. are not usually employed, artifacts resulting from these comparatively harsh treatments are avoided, and a relative advantage lies with the use of the electron microscope.

On the other hand the instrument has several disadvantages, one of the more serious being the need for complete drying of the object being studied. Water and other liquids strongly absorb electrons and must be eliminated from the system in order that the object structures will become visible by contrast. Likewise atmospheric gases cannot be present for they absorb and scatter the electrons, so the electron source, object, lenses, and photographic system must all be maintained in a high vacuum.

Artifacts of drying then may result and often appear to be serious. For example a bacterial cell may be collapsed or may shrink during drying until its true three dimensional character is largely lost. In addition small particles tend to aggregate around large ones apparently as a result of the drying process. Finally small objects attached to larger ones, filamentous appendages, or the internal contents of cells may be separated from the parent objects, stretched out of shape, or even destroyed by the large surface forces that operate during the drying of the specimen.

There are indications that the drying artifacts may be completely eliminated. Anderson has suggested that these various artifacts arise during the evaporation of liquids because of the passage of a gas-liquid phase boundary through the preparation, and that the large surface forces existing at the phase boundary distort any structures encountered.

To avoid subjecting cells and the like to a moving phase boundary during dehydration, Anderson has replaced the water stepwise with a series of completely miscible liquids, first acetone which is replaced in turn with amyl acetate, and the latter finally by liquid carbon dioxide. The purpose of using liquids completely miscible with both the preceding and following liquids is to avoid possible damage at liquid-liquid phase boundaries which might occur if incompletely miscible substances were used. Since the last liquid exchange step involves carbon dioxide, it is carried out under enough pressure to permit the use of the carbon dioxide in liquid form. Finally the specimen wet only with liquid carbon dioxide under pressure is brought above the critical temperature of carbon dioxide,  $30.8^{\circ}\text{C}$ . Above the critical point (at say  $40^{\circ}\text{C}$ .) the liquid phase cannot exist so the transition from liquid to gas does not involve the formation of a phase boundary. When the gaseous carbon dioxide is released from the warm system, the specimen has been dried without damage from surface forces and is ready for use.

The above procedure for drying has led to a great improvement in the quality of specimens prepared for examination with the electron micro-

scope, and many small structures, e.g. flagella, have been photographed for the first time in something like their natural positions and dimensions. An important adjunct with such a method of preparing specimens is the use of stereoscopic photographs for observation and study. A combination of drying above the critical point and stereoscopic pictures yields full three dimensional perspective for interpretation of structure and is much superior to ordinary photographs taken after conventional drying of specimens *in vacuo*.

Another limitation in electron microscopy is the strong absorption of electrons by most materials and the subsequent concealment of internal structures due to the complete opacity of the object. This difficulty may be overcome to some extent by decreasing the wavelength of the electron beam which increases its penetrating power. This procedure has permitted the resolution of such internal structures as postulated bacterial nuclei. However, other similar attempts are recorded in which cells change from uniformly opaque to uniformly semitransparent bodies without resolution of any internal structure. In other words the transmission of electrons is essentially equivalent for many materials and such materials therefore cannot be differentiated. A possible corollary of the preceding limitation is the present inability of the electron microscopic techniques to yield information of the chemical composition of the structures demonstrated.

On the other hand, if cells are suddenly exposed to an electron beam of high intensity various cellular components may be altered. The variable extent of such disruption is a valuable clue as to differences in structure. Also because the disruption is accompanied by a decreased resistance to the passage of the electron beam clearer observation of certain interior structures may be possible.

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## CHAPTER V

# Dyes and Staining

The instrument most used in the study of bacteria has been the ordinary light microscope. As is well known, living bacteria in the usual suspension media do not show much structure or detail because the various refractive indexes in the system are not sufficiently different. Hence it is customary to resort to staining procedures. This process implies adding color contrast to the existing optical contrast and thereby greatly increasing the visibility of small structures.

Historically the growth of knowledge of the cytology of bacteria has been highly dependent upon the use of staining procedures. It is significant that the application of newer techniques and instruments has not disturbed the knowledge of structure obtained by the traditional staining methods, but rather has confirmed and extended this knowledge. Unquestionably, staining methods though yielding a rich harvest in the past have not yet had their potentialities fully explored and exhausted. Much may still be expected from those investigators with an understanding of the principles of staining who imaginatively apply this knowledge and, cognizant of the limitations of even the best techniques, can couple their enthusiasm with restraint in interpretation.

### LIGHT AND COLOR

The radiation commonly called light is a part of the class of energy known as electromagnetic radiation. This class consists of a continuous spectrum or unbroken series with one arbitrarily defined type of radiation merging into the next. All electromagnetic radiations have a number of common properties, two being transmissibility through a vacuum and obedience to the relation,

$$\lambda \nu = c \quad (1)$$

where  $\lambda$  is the wave length,  $\nu$  is the frequency (number of waves per second), and  $c$  is the velocity in the medium. The velocity is the same for all wave-lengths in vacuo and is approximately  $3 \times 10^{10}$  cm. per sec.

### *The Nature of Color*

Usually the term light is limited to that portion of the spectrum which stimulates the optic nerve and thus produces the sensation known to us as

seeing. This "visible light" includes the electromagnetic radiations lying within the wavelength range of approximately 400 to 700  $m\mu$  (1  $m\mu$  =  $10^{-7}$  cm.). The extent and nature of the sensation resulting from exposure of the eye to light varies among individual observers. It also depends upon the intensity and wavelength of the incident radiation. The wavelength effect is manifested as the sensation known as color and results from a different optical response in the physiological system as the wavelength is changed. One theory proposes that in the eye there are three different light-sensitive components which have different wavelength responses. For example, one material is thought to be especially sensitive to blue light and much less so to red and green, while the others are sensitive to red and green respectively and relatively insensitive to the other spectral colors. In a general way then, the color sensation is the sum of a strong response by a particular sensitive material and weak responses by the other sensitive components. The nerve connections and the brain interpret this specific response as a color. Variations in hue result because the sensitivities to a wavelength are not clear cut but change gradually, thereby permitting a steady decrease in the stimulation of one component with an increase in another. Uniform illumination by the entire range of visible wavelengths results in a uniform physiological response, and the light is then said to be white.

Intermediate hues perceptible to the eye arise from either of two sources. Many shades appear in the visible spectrum as lights of characteristic wavelengths and are considered to be *pure colors* since they cannot be separated into two or more component colors. Most of these same shades and a number of others may also be obtained by combining the pure colors in various ways. For example, a certain combination of red and green lights will produce a yellow light that the eye cannot distinguish from a monochromatic (spectrally pure) yellow. Among the *non-spectral colors* available by mixing pure lights might be cited purple which is a mixture of violet and red lights and corresponds to no single wavelength of the visible spectrum.

### *Properties of Colors*

There are three common characteristics possessed by a color: *hue*, *saturation*, and *luminosity*. The *hue*, of course, depends upon the wavelength if it is a pure spectral color or otherwise upon the wavelengths of its components. Table 3 correlates the wavelength of light with its color for the major hues of the visible spectrum. The values given are only approximate since a range of hues corresponds to a range of wavelengths, and the matching is quite subjective.

The *saturation* of a color is determined by the amount of white light the



color contains and is called saturated when entirely free of white light. Pink and pale blue are examples of common unsaturated colors. *Luminosity* is a measure of brightness, or perhaps more properly the degree of visual response to a color. The eye detects yellow and green wavelengths best with orange and blue next and red and violet least well. In other words a given small quantity of radiant energy is most easily detected by the eye when its wavelength is near the center of the visible spectrum. These wavelengths are said to be highly luminous.

TABLE 3

*Wavelengths of visible light, the corresponding colors, and the complementary colors*

WAVELENGTH	CORRESPONDING COLOR	COMPLEMENTARY COLOR
<i>mμ</i>		
400	Violet	Greenish yellow
430	Indigo	Yellow
460	Blue	Orange
490	Blue-green	Red
510	Green	Deep red
530	Yellow-green	Violet
560	Yellow	Indigo
600	Orange	Blue
640	Red	Blue-green
710	Deep red	Green

### *Origins of Colored Light*

Colored light arises in three possible ways. An object may be luminous or glow for one of several reasons and will emit light having definite wavelengths. This emitted light may be white but it often is limited in wavelength to some particular part of the spectrum and appears colored. Secondly, white light may pass through a transparent object and emerge colored. Many clear materials possess the property of absorbing light of certain wavelengths while permitting other wavelengths to pass on through. When illuminated by white light a ruby absorbs a high proportion of the shorter wavelengths, blue and green, and allows the others to emerge yielding red light. If an absorbing body removes a given wavelength from white light the light remaining is a colored mixture of the residual wavelengths and the color is said to be *complementary* to the absorbed color. The absorbed color and its complement are both present in white light, and, furthermore, if they are combined white light results. Table 3 includes some "pure" colors and their complements.

In bacteriology such transmission colors are very commonly encountered either in natural organisms which selectively filter out part of the spectrum

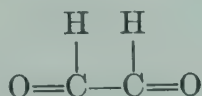
or in stained preparations to which a selective filter has been added. The presence of color "in the object" is of great advantage in microscopy, for the contrast in light intensity between the object and its medium is greatly supplemented by color contrast. The visibility of small objects then becomes much increased.

Finally, colored light may arise from an opaque article by reflection. Reflection by opaque bodies, usually solids, involves some sort of reaction of the incident light with the object, perhaps in the manner of a very shallow penetration. The light then apparently emerges from the surface of the object and is propagated in a new direction. Materials that appear colored by reflected light have the power of absorbing part of the incident intensity and reflect only the unabsorbed wavelengths thereby giving rise to the sensation of color. As an illustration, the foliage of ordinary plants is green because the incident white sunlight is almost completely absorbed except for the reflected middle wavelengths causing the response in the eye that we call green. It will be apparent that green leaves illuminated with red or blue light or both will appear black because the incident light will be completely absorbed when there is no green light to be reflected. In a similar connection grey would result from the general but incomplete absorption of white light with a weak but general reflection by the surface in question. Black denotes complete absorption of the incident light whatever its wavelength may be. Black cannot, of course, be formed by mixing lights but occurs only when all light is removed, and the eye then does not receive a stimulus.

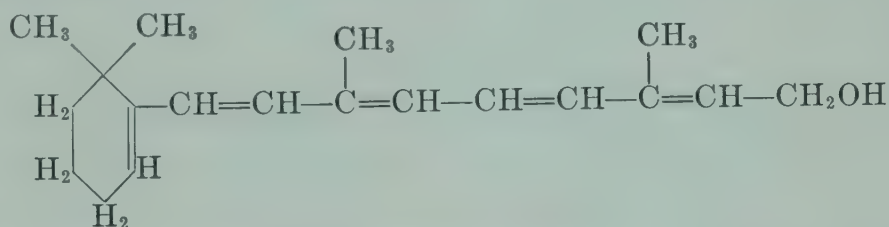
### *Light Absorption*

Molecules always absorb parts of the general spectrum of radiation although this absorption frequently does not fall within the visible range. In such instances the molecules are colorless as distinguished from the smaller group of molecules which absorb part of the wavelengths in the visible spectrum. The phenomenon of absorption in the visible and ultra-violet regions of radiation is associated with the outer or valence electrons of the atoms in the molecules. These electrons absorb radiant energy and in so doing are brought to a higher energy state. The changes involved in this process follow discontinuous procedures which are dependent upon (1) the energy of the light absorbed (energy =  $h\nu$  where  $h$  is Planck's constant and  $\nu$  is frequency), (2) the nature and energy states of the individual atoms, and (3) the number and arrangement of those atoms in the molecules. It is found that characteristic bands of wavelengths will be absorbed by a molecule and that these absorption bands are separated by intermediate ranges of wavelength which are absorbed less strongly or not at all.

Firmly bound electrons do not absorb low energy light (long wavelength) and can be moved to higher energy states only by the high energy light in the ultraviolet range. More mobile electrons will absorb visible light and give rise to colored compounds. Organic molecules containing only covalent single bonds like those in the paraffin hydrocarbons absorb only short ultraviolet wavelengths in the range affecting outer electrons because all the electrons are in stable states in such molecules. On the other hand when unsaturation (resulting in more mobile electrons) exists in the structure, absorption occurs at longer wavelengths, and as the unsaturation increases the wavelengths absorbed also increase. *Conjugation of unsaturation*, the terminology applied to a system of alternating multiple and single bonds, is especially effective in raising the wavelength of the light absorbed. Such a system may be illustrated by glyoxal which is probably the smallest colored organic compound. It is pale yellow and has the conjugated structure,



Unsaturation may involve only carbon atoms as in the case of vitamin A, also yellow,



or other atoms like *O*, *N*, *S* in addition to the carbon. As a general rule these latter atoms shift the absorption to longer wavelengths when they replace unsaturated carbon in a molecule (see Table 4). In Table 4 it is evident from the example of azobenzene which absorbs blue light (450 mμ) that the complementary mixed color remains and gives the compound its characteristic appearance. This situation maintains for all the structures having a single narrow absorption band in the visible spectrum, and in these cases the color of the compound can be predicted from knowledge of the wavelength range absorbed. Conversely, suggestions of the absorption characteristics of such a substance may be drawn from its color by means of Table 1. However, complex molecules often show two absorption bands, and therefore it is not always possible to deduce the wavelengths absorbed from the color of the compound. For example, a substance may be green because it has a band in the deep red region or because it has two bands, one in the indigo blue and one in the orange.



## THE GENERAL CHEMISTRY OF DYES

A *dye* has been defined by Cohn as an "organic compound which contains chromophoric and auxochromic groups attached to benzene rings, the color being attributable to the chromophores and the dyeing property to the salt-forming auxochromes". In other words this definition states that a dye is a colored aromatic compound having the ability to form a salt with

TABLE 4

*Effect on the absorption of light brought about by replacement of carbon by nitrogen*

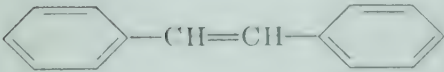
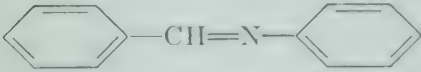
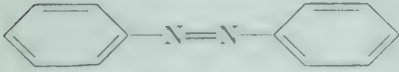
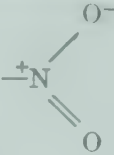
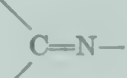


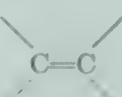
STRUCTURAL FORMULA	NAME	WAVE-LENGTH OF MAXIMUM ABSORP.	COLOR OF THE COMPOUND
	Stilbene	$m\mu$ 295	None
	Benzalaniline	330	None
	Azobenzene	450	Orange

TABLE 5

*The structures and names of common chromophoric groups*

GROUPS POTENT IN THE PRODUCTION OF COLOR		LESS POTENT GROUPS CONTRIBUTING TO COLOR PRODUCTION	
Structure	Name	Structure	Name
$-\text{N}=\text{N}-$	azo		nitro
$-\dot{\text{N}}=\text{O}$	nitroso		imino
$-\text{N}=\text{N}^+-\text{O}^-$	azoxy		carbonyl
	thio		ethenyl

some substrate. However, it has been customary to describe as dyes many types of compounds which are not covered by the above definition. In the first place, aromatic rings need not be present at all, and often the aromatic rings are incidental to the structures apparently responsible for the color.

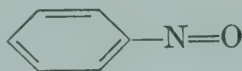
Dyes are colored of course since their use depends upon this property and certainly may be said to possess chromophoric groups. Yet salt-forming groups are not always required, for the dyeing of some materials does not involve salt formation, and dyes are used with which salt formation is either unlikely or impossible. More generally then a *dye* may be *any colored compound that reacts with, is adsorbed by, or dissolves in another phase and renders that phase colored*. The phase so altered is usually but not always a solid. The so-called fat stains used in biology are employed to make fat droplets discernible and will color both liquid and solid phases.

### Color Producing Structures

The term *chromophore* is applied to the structural grouping which imparts the color to the dye molecule. Usually more than one chromophoric group is needed to give rise to color in a substance and rather large numbers may be present in ordinary dye molecules. Table 5 includes some of the more common chromophoric groups. They may be combined in an enormous variety of ways to yield colored substances, e.g.,

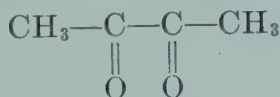


*p*-Benzoquinone  
(yellow)

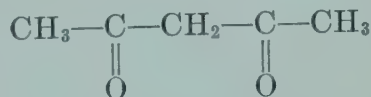


Nitrosobenzene  
(pale green)

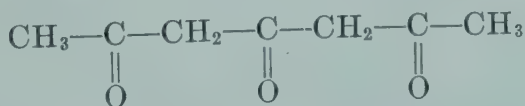
Although the bonds in an aromatic nucleus like those in the phenyl group of nitrosobenzene do not compare in effect with ethylenic double bonds, nevertheless they do contribute to the color of the molecule. The maximum effect on color is obtained from the chromophoric groups present when they are conjugated with one another. For instance biacetyl is yellow while acetylacetone and even diacetylacetone are colorless:



Biacetyl (yellow)



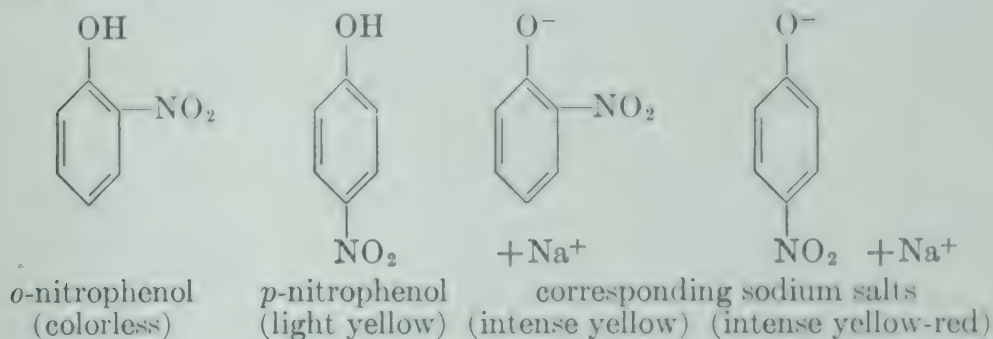
Acetylacetone (colorless)



Diacetylacetone (colorless)

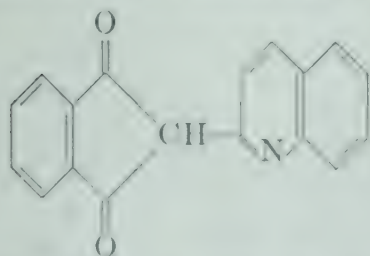
### Auxiliary Structures

There are certain other substituent groups which do not in themselves produce color in a molecule but which do augment the effects of chromophoric groups. Such auxiliary structures are known as *auxochrome* groups and are listed in the decreasing order of effectiveness:  $-\text{NR}_2$ ,  $-\text{NHR}$ ,  $-\text{NH}_2$ ,  $-\text{OH}$ ,  $-\text{OCH}_3$ ,  $\text{I}$ ,  $\text{Br}$ ,  $\text{Cl}$ . While it is obvious that the last four groups cannot owe their auxochromic effect to salt formation, it is now recognized that the powerful influence exerted under certain conditions by the hydroxyl and the various amino groups does involve salt formation. This effect is illustrated by:

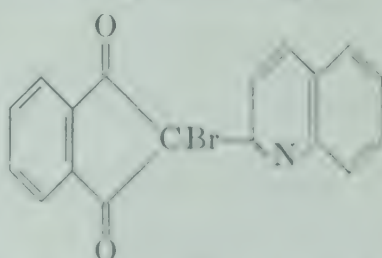


While salt formation involving amino and hydroxyl groups does affect the color, not all salt forming groups are important in their influence on color. For instance sulfonic acids, carboxylic acids and quaternary ammonium bases form salts readily. The first two are often used in the form of salts to increase the solubility in water, but they have little or no action on the color.

A number of dyes are used under conditions in which salt formation would be unexpected, and yet these dyes show color that cannot be explained by ordinary structural formulae. One type apparently involves the formation of inner salts. These dyes possess the high melting points characteristic of salts and colors ordinarily associated with extensive conjugation and powerful chromophoric groups. The following structures serve to illustrate the logic concerned in one of these "abnormally" intense dyes:

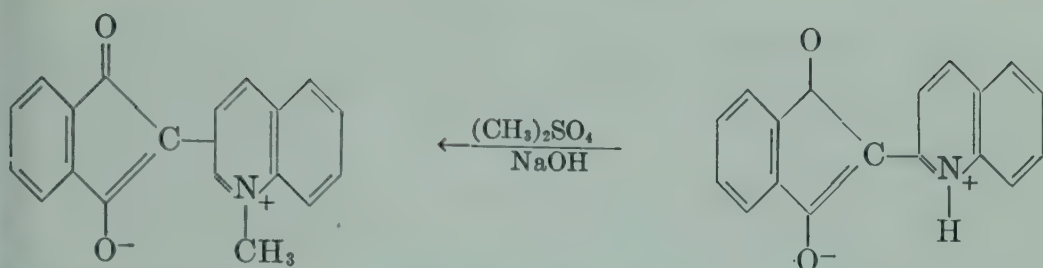


Conventional structure of quinoline yellow. No color expected with the limited conjugation present. Solubility in alkali not expected.



Bromoquinoline yellow (colorless). Conjugation definitely prevented between the left and right portions of the molecule.

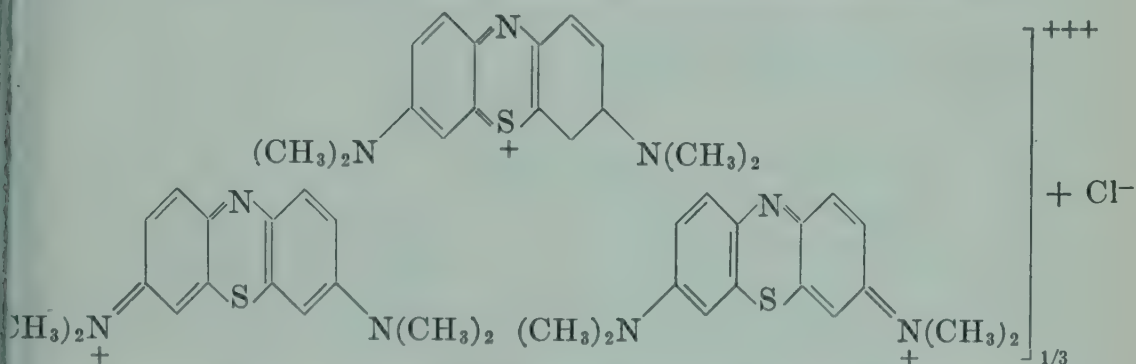




N-Methylquinoline yellow (yellow, m. p.  $240^{\circ}$ ). Location of the methyl group requires the formation of a dipolar ion.

Dipolar structure of quinoline yellow. The actual dye has the same color as N-methylquinoline yellow, m.p.  $252^{\circ}$ . Should be and is alkali soluble.

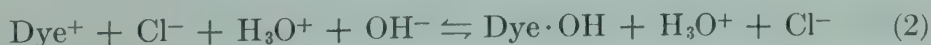
The theory of resonance contributes to a knowledge of color formation in such dyes as methylene blue which involves the three structures:



These formulas do not represent molecules in a dynamic equilibrium with each other, rather the actual substance is composed of molecules that are all alike and have a structure that is a composite of the three contributing forms. In other words the properties of methylene blue are the properties of a fixed, average molecule. This resonance hybrid is more intensely colored than would be any single one of the participating structures.

### The Dye Salts

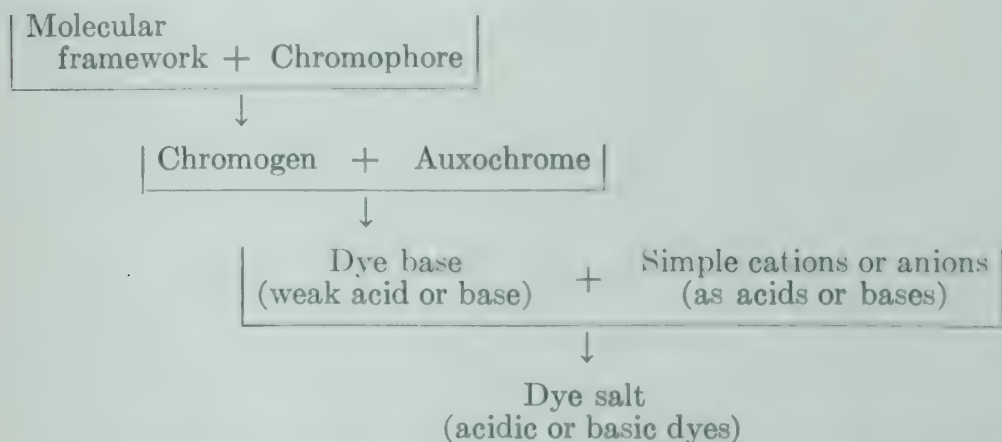
The many dyes used as salts are subdivided into *acidic* or *basic* dyes depending upon the nature of the charge on the dye ion. *Basic dyes* are those dye salts in which the dye ion is a cation (positively charged). The common anions in such dyes are chloride, sulfate, acetate, and oxalate. Aqueous solutions of many basic dyes are slightly acidic by hydrolysis as illustrated by means of a dye chloride:



A similar hydrolysis will occur with the basic dye acetates, but since acetate also hydrolyzes removing  $\text{H}_3\text{O}^+$  from the system, the final pH of the solution will depend upon the relative hydrolyses of dye cation and the acetate.

Conversely, the *acid dyes* are salts of metal cations and dye anions. The cations commonly used are sodium, potassium, calcium, and ammonium. The first three form strong bases, and their dye salts commonly hydrolyze to yield weakly alkaline aqueous solutions by a process similar to that in the reaction above. In common with other typical salts, the dye salts themselves are completely dissociated in both the solid and solution states.

To illustrate the confusion existing in the nomenclature of dyes and stains one may cite the term basic dye. Basic dyes are not alkalis but are salts, and they often actually form acid solutions as indicated in the general hydrolysis reaction above. Also it is customary to describe the colored, unionized molecule from which a dye salt can be prepared as a *dye base*. Actually such substances may be either weak bases or acids. The following diagram summarizes the nomenclature:



Dye bases often have a radically different solubility than the dye salts and are usually less water-soluble and more soluble in organic solvents. As a matter of fact, fat dyes useful for staining nonpolar materials may be prepared simply by adding sodium hydroxide to the aqueous solutions of certain basic dyes. The weakly basic dye base then precipitates from solution. Common dyes such as methylene blue, basic fuchsin and neutral red may thus be used to prepare fat soluble dye bases.

### *Compound Dyes*

On mixing solutions of acid and basic dyes there can be combination of the colored anions and the colored cations by simple metathesis. The extent of this reaction depends upon the solubility product of the new salt, and this in turn depends upon its nature. Since the component ions are derived from weak acids and weak bases, the ions will be extensively hydrolyzed when the dye is dissolved in water, hence the aqueous solution will consist largely of the two dye bases. This type of dye is known as a *compound dye*.

by the chemist and as a *neutral stain* by the biologist. The former term is probably preferable since there are two colored ions in the molecule in contrast to the one in ordinary dye salts, and the dye may or may not yield a neutral solution in water. Speaking generally, the compound dyes are less soluble in water than are either of the parent dye salts.

The staining properties of compound dyes are often sufficiently different and selective so as to recommend them for special purposes. They have found their greatest utility as blood stains. Historically the first such blood stain was the Romanovsky stain, a combination of eosin with methylene blue. However, the water insolubility of compound dyes sometimes presents difficulties in their use. This factor has been overcome in various ways, for example, by mixing the acid and basic dyes just before use, by adding a slight excess of either acid or basic dye which increases the solubility of the compound dye, by adding a water soluble organic solvent, or by staining with one dye and then adding the other dye in which case the compound dye forms within the material being stained.

### *Commercial Dyes*

Dyes are ordinarily sold as the salts. If not they are referred to on the label as the dye base, e.g., fuchsin, base. Commercially available dyes are rarely pure substances, nor are separate batches of a dye from the same or different manufacturers always equivalent. They may consist of mixtures of related dyes as well as of appreciable quantities of non-dye materials such as mineral salts. Inasmuch as the histological qualities of dyes are much influenced by the nature of impurities there has been an organized effort made to certify individual dye batches on the basis of their usefulness in certain standard staining procedures. An independent organization, the Biological Stain Commission, which includes representatives of cooperating American scientific societies seeks to "gather information concerning the nature of dyes as related to their use in microscopic technic", and "by working with manufacturers and dealers it endeavors to see that the supply of stains available in America is of the highest possible quality as judged by their performance in actual laboratory use". Batches of particular dyes submitted to the Commission are tested and if found satisfactory are issued a certification number. Containers of certified dye will include a statement of the purity of the product and have the Commission's label with the certification number. Insofar as possible biologists are well advised to use only certified dyes and to include in their reports the certification numbers of the dyes employed.

There is no agreed upon system for naming individual dyes, and a given product may be known by a variety of names. There are available two indexes of dyes, one published in Germany, the *Schultz Farbestofftabellen*,



and the other in England, the *Colour Index of the Society of Dyers and Colourists*. Commercially packaged dyes usually include the Colour Index number (C. I. No.) on the label.

### *Leuco Dyes*

The terms leuco-dye or leuco-compound are common in connection with the dyeing of fibers and are occasionally encountered by the bacteriologist. A leuco-dye is ordinarily a reduced form of a vat dye and is soluble in the dye bath. It usually is colorless since the unsaturation has been diminished and the conjugation interrupted. The reduction often involves a quinoid structure, and the reduced form is readily reoxidized on the fiber to the colored, insoluble dye. Naturally the leuco-form must have some affinity for the material being dyed in order that it will be retained until the oxidation is complete.

The use of leuco-compounds is commonplace in the study of cell respiration. For example, methylene blue retains its blue color in the presence of oxygen or in the absence of reducing systems. If a suspension of cells respiring in the absence of oxygen contains methylene blue the dye acts as a hydrogen acceptor for the dehydrogenases of the cells. In so doing the methylene blue is reduced to a colorless leuco form, and the extent of the reduction is a measure of the dehydrogenase activity present. In addition to an important role in work on cellular metabolism such leuco-dye systems may be employed in the growth cultures of organisms to indicate or even to provide favorable oxidation-reduction potentials for maximum growth.

Various leuco-triphenylmethane dyes have been employed for detecting bacterial polysaccharides since some of the leuco-dyes are precipitated by the complex carbohydrates present and others are oxidized to colored forms. Many other components of the cells give no evidence of such reaction.

### *Dichromatism*

*Dichromatism* is a phenomenon observed in the solutions of some dyes and colored compounds. A thin layer of a colored solution may appear to be green while a thicker layer is reddish. This peculiar property of some materials in solution is called dichromatism, and such materials are said to be *dichromatic*. Substances behaving in this way possess absorption bands in two different portions of the visible spectrum. These bands lie in regions differing in luminosity (eye sensitivity) and differ in their intensity of absorption. In the case mentioned, the compound absorbs strongly in the blue and has a weaker absorption in the yellow, orange, and near red. It, therefore, transmits some of both green and deep red with more of the former being absorbed. Small thicknesses allow passage of sufficient green

to cause a net optic response of green because the eye is much more sensitive to green than to deep red. However, in the thicker layers of solution the green is so completely absorbed that the eye cannot respond to the very slight amount of green light transmitted, and the deep red produces the color response because it is now transmitted to a very much greater extent. The complete phenomenon then becomes a case of two opposing effects, one controlling the visual reaction under one set of conditions and the other predominating under other conditions.

It will be immediately apparent that a mixture of two pigments can reproduce the effects described above for the solution of a single substance. Hence, various mixtures of dyes and stains as well as pure stains may sometimes lead to artifacts of color. Furthermore, stained objects may be one color when viewed individually but clumps or masses of the same objects may be a different color owing to an increase in the thickness.

### *Metachromasy*

Another phenomenon that involves a change in the color of a system is called *metachromatism* or *metachromasy*. The color change results from a change in the concentration of the dye or from the presence of certain materials which affect the state of dispersion of the dye. Sometimes the effect on the absorption spectrum is plainly visible to the eye as a change in color but at other times is evident only on the quantitative absorption spectrum.

Many dyes, particularly including those of the thiazine and oxazine classes, do not obey Beer's Law<sup>1</sup> when dissolved in water. For example, methylene blue  $6 \times 10^{-5}$  molar in water has an absorption maximum at 660 m $\mu$ . At a concentration of  $6 \times 10^{-4}$  the same maximum still exists, but the absorption on a unit basis is much reduced, and a second and higher maximum is present at 610 m $\mu$ . At still higher concentrations this shift is even greater.

Metachromasy of this type is attributed to association of the individual dye molecules. In very dilute solution the spectrum is typical of the dye monomers. As the concentration increases the monomers pair to dimers which have a slightly shifted absorption maximum as illustrated above with methylene blue. Even greater association occurs at higher concentration, and a third band appears which is probably a composite of the bands from molecular aggregates of various sizes.

In alcoholic solution this type of molecular association apparently does not occur since only one absorption maximum appears, and the solutions obey Beer's Law. The presence of surface active agents has a similar effect

<sup>1</sup> Beer's Law is discussed in the Appendix.

and, at least in appreciable concentration, keeps the dye dispersed into the individual molecules.

On the other hand, fairly high concentrations of salts tend to salt out or coagulate the monomers into larger species and often produce great spectral shifts. Many large biologically occurring molecules produce a similar result.

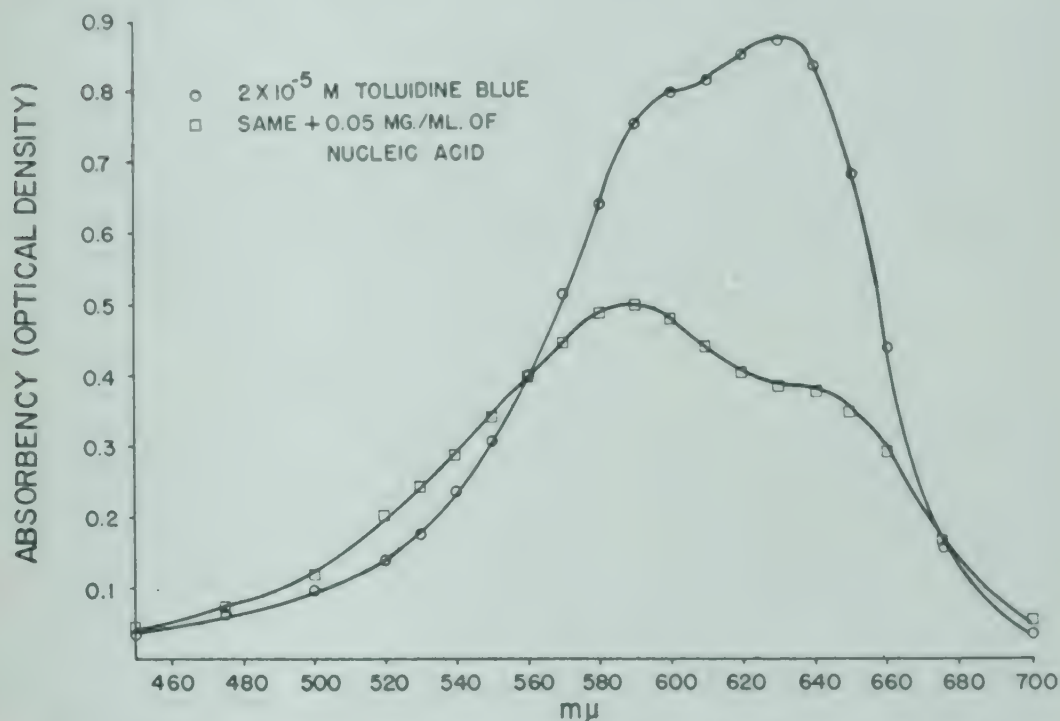


FIG. 14. Illustrating the metachromasy of nucleic acid. Observe the shoulder on the curve for the dye alone. This elevated absorption at about 600  $m\mu$  indicates that some of the dimeric form of the dye exists at this concentration in water. When ribonucleic acid from yeast is present (as a salt) the total absorption decreases with the maximum at 590  $m\mu$  becoming the most important region relatively. Not all of the dye aggregates as indicated by the shoulder at 630  $m\mu$ . The visible color changes from an intense blue for the dye solution to a less intense reddish purple for the solution containing both the nucleic acid salt and the dye.

apparently acting as centers for the coagulation of the dye. Nucleic acid, complex metaphosphates, large carbohydrate esters of sulfate, agar, and the like shift the spectrum of the metachromatic dyes. (See fig. 14.)

Certain of these large molecules in cells may be routinely identified and located by metachromatic staining. This process depends on the use of one of several dyes exhibiting metachromasy. The nucleic acids, chondroitin sulfuric acids, etc. stain a color different than that of the dye used and thereby are revealed by a color contrasting with that of the rest of the structure.



Since the apparent color of a dye may not reveal the presence of differently colored impurities, objects may sometimes take up the impurity preferentially and show an unexpected color. Though these instances are not truly due to metachromasy, they are known by that name in bacteriology. The situation may be illustrated using a color base and its dye salt which do not have the same colors. If both are present in the staining solution, different cell structures may stain different colors. Cells are stained in such a way with Nile blue in alkaline solution; the fats take up the color base and appear red while the cytoplasm takes up the dye salt and becomes blue. Similarly, volutin granules stain red with alkaline methylene blue. Here, however, some methylene blue is atmospherically oxidized to methylene violet at the high pH, and the latter selectively stains the volutin.

### INDICATORS

Owing to their very widespread use, indicators warrant consideration in connection with dyes since many dyes are indicators. In a general way the term *indicator* is applied to any device or system denoting the quantitative state of another system. In the physical and biological sciences the term is most often applied to a thing which indicates the endpoint of a quantitative measurement. The indicator may be a group of mice used in a bacterial toxin titration or a dye employed in the estimation of the hydrogen ion concentration of a solution. Our present concern is confined to the so-called chemical indicators of the second class.

An indicator must change as some property of the measuring system changes, and the change in the indicator must be evident to the experimentalist. This requirement for a visible change means that the color, turbidity, surface tension, or some other easily measured quantity must vary. Since color changes are by far the most widely used, the discussion will be limited accordingly to colored chemical indicators.

#### *Colored Chemical Indicators*

One of the simplest kinds of laboratory indicators serves as an index of moisture. Cobaltous chloride ( $\text{CoCl}_2$ ) is a blue hygroscopic solid which readily sorbs water from a moist environment to form certain colorless intermediates and finally a solid red hydrate ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ). During the hydration process the color changes from deep blue through pale blue and pink to red depending upon the relative amounts of blue, colorless, and red compounds. Materials of this sort find wide application as moisture indicators where changes are slow enough to allow the relatively slow hydration process to keep pace with the system. Usually a little of such a substance is mixed with a cheaper but colorless drying agent and retains the color of its dry form until the principal component has become partly hydrated.

Titration indicators are indispensable to many methods of volumetric analysis and may be divided into two classes, the *external* and the *internal* indicators. The former often involve the use of indicator materials which react with the system to form colored substances, but they may consume a portion of one of the reactants and in so doing would displace the endpoint. Hence, in such a case the indicator must be kept separate from the principal part of the reaction system. Small increments of this latter are added to aliquots of indicator solution at intervals during the titration, and the endpoint is reached when a desired change takes place in the color of the test sample of indicator. Since the titration must necessarily be discontinuous and many manipulations are involved, the external indicators are often not desirable for the routine analysis of large numbers of samples. However, they do find wide use especially when the final system is to be put to some purpose wherein the presence of an indicator is not permissible. Very often biological materials or solutions for injection require adjustment of pH or oxidation-reduction potential, and external indicators are ideal under such circumstances.

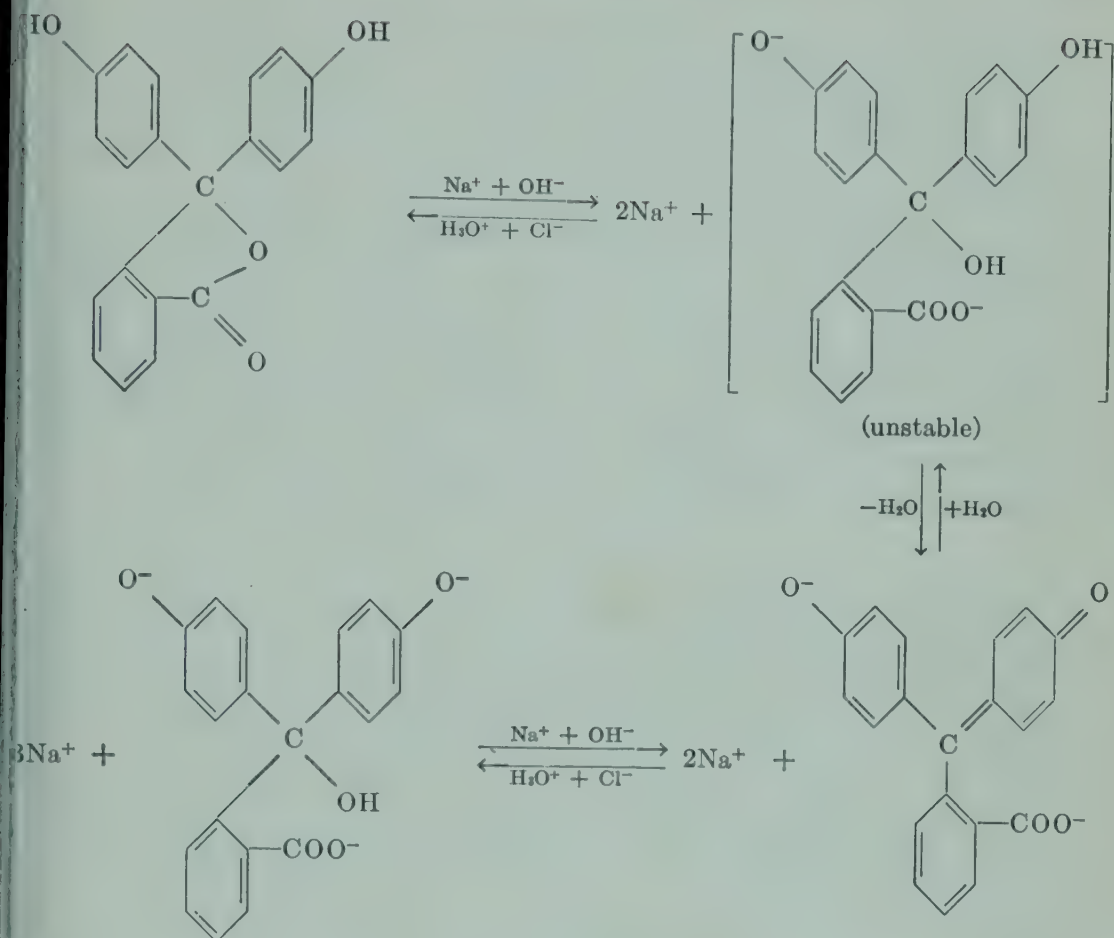
Internal indicators are placed in the system as the name implies, but they must not cause any significant change in the nature and extent of the measuring reaction. Typical uses are for indicators which reveal particular hydrogen ion concentrations, oxidation-reduction potentials, or the appearance of appreciable concentrations of certain ions.

### *Hydrogen Ion Indicators*

pH indicators are organic compounds that ionize at characteristic hydrogen ion concentrations and show different colors in the ionized and unionized forms. Such indicators are, of course, weak acids or weak bases and are titrated through a color change just at the completion of the titration of the reactant with which the indicator is mixed.

Phenolphthalein may be used as an example of a pH indicator. It is a weak colorless acid which forms a colored salt in alkaline solution. The reaction may be represented as shown in the formula at top of next page. Aqueous acid reverses all of the above reactions as shown, and the acid form of phenolphthalein results. The formation of a colorless monosodium salt is actually the first step in the series but has been omitted from the above scheme. The red disodium salt begins to appear at pH 8.0, and the reaction is complete at approximately pH 9.8. At higher pH values the color disappears as the trisodium salt is formed. At a given concentration of phenolphthalein the color depends upon the relative amounts of colorless and red forms and is greatest when the maximum quantity of disodium salt is present.

Since the color changes continuously in intensity over a rather broad



Trisodium salt (colorless)

Disodium salt (red)

range of hydrogen ion concentration, titration must be stopped at some reproducible color. In the case of this particular indicator the first definite appearance of pink is best because subsequent changes are limited to intensity changes which are less readily differentiated than are qualitative differences between colors. When titrating with acid the color will fade and the sudden disappearance of color is probably the most easily detected stage.

### *The Use of pH Indicators*

There are several important points which should be considered when using pH indicators. A small, definite amount of indicator should be used since the indicator does consume a small amount of titrating agent at the end-point, and this effect must be kept constant and minimal. It may then be corrected for by means of indicator blanks. These blanks will also measure the quantity of reagent required to bring the working volume of water to the pH at which the indicator change occurs.



Other things being equal the indicator with the greatest color contrast will be the most accurate especially with dilute solutions. Stock indicator solutions are ordinarily prepared for convenience, and since alcohol is often used for reasons of solubility (as with phenolphthalein) occasionally such a solvent may have an undesirable effect.

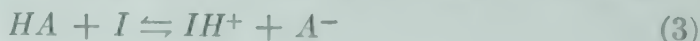
Most important of all the general points to be considered is the selection of the proper indicator for the pH of the endpoint. This choice implies knowledge of the pH of the equivalence point (the pH at which the reaction is quantitatively complete). If the pH of the indicator change differs from that of the equivalence point, then too much or too little of the titrating reactant will be added. Tables of indicators with the colors involved and the pH interval corresponding to the color change are available in text books of analytical chemistry and in handbooks.

### *Protein Effect on Indicators*

The so-called protein error involved in the use of indicators arises because proteins often have powerful tendencies to adsorb other molecules. When an indicator is used in solutions of proteins, it is commonly observed that much of one form of the indicator is adsorbed while most of the other form remains in solution. This differential adsorption leads to an abnormal shift in the ionization by withdrawing a part of one form. The ionization of the indicator, therefore, must shift to satisfy the equilibrium constant, and the indicator will change color at a pH different than that in ordinary solution.

### *The Effect of Dissolved Salts on Indicators*

Another difficulty arises when salts are present, and it results from a lowering of the activity coefficients of the ions involved. The equilibrium of a weak acid, HA, and a weakly basic indicator, I, may be written as:



The equilibrium constant is frequently written in the form:

$$K = \frac{[IH^+][A^-]}{[HA][I]} \quad (4)$$

where the brackets denote the molar concentrations. Actually this expression applies only to the idealized situation of great dilution and the absence of other ions.

More generally this equation should be modified to:

$$K = \frac{f_{IH^+}[IH^+]f_{A^-}[A^-]}{f_I[I] f_{HA}[HA]} \quad (5)$$

The factors indicated by  $f$  are the activity coefficients for the ions indicated by the subscripts, and these coefficients adjust the actual molar concentrations to effective concentrations. They correct for such things as the association of particles and interactions with the solvent and with other substances present. In other words, activity coefficients correct for the non-ideality of behavior, and they approach unity as an upper limit in very dilute solutions of single materials.

When extra ions are added to a system of this type the activity coefficients of the charged particles involved are all lowered. Evidently this lowering results from the increased opportunity for association of oppositely charged ions. Each ion seems to have a statistical tendency to surround itself with ions of opposite charge. Such a process restricts the independence of each ion somewhat and thereby reduces the effective concentration of every ionic species.

The equilibrium constant  $K$  of equation (5) does not vary. Yet  $f_{\text{IH}^+}$  and  $f_{\text{A}^-}$  decrease while  $f_{\text{I}}$  and  $f_{\text{HA}}$  are essentially unchanged. Consequently  $[\text{IH}^+]$  and  $[\text{A}^-]$  must increase slightly, shifting reaction (3) to the right and slightly reducing the concentrations of  $\text{HA}$  and  $\text{I}$ . As a result the indicator changes color too soon, and the pH is lower than it should be when compared to the endpoint in a salt free system. Hence, if an appreciable concentration of salt is present in certain types of indicator titrations of weak acids, correction should be made by adjusting the salt concentration of the indicator blank.

It will be clear that a similar development can be made with other types of indicators (weakly acidic) and in the titration of weak bases. Of the four possible combinations of indicators and weak acid or base titrations, two yield important salt effects and the other two do not. In the latter cases one ion appears in the numerator and one in the denominator of the expression for the equilibrium constant. Since the activity coefficients of all ions are similarly affected by added salt, the reductions in the coefficients cancel each other out of the equilibrium constant.

#### *Alcohol and Carbon Dioxide Effects on Indicators*

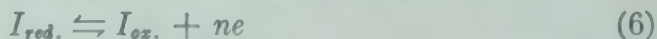
Added alcohol lowers the dielectric constant of aqueous solutions and increases the activity coefficients of the ions present. In the instance developed above, the reaction would be shifted to the left, and the endpoint would come at a higher pH than in water alone. This reasoning applies to the increase in pH caused by the addition of alcohol to aqueous solutions of weak acids or acid buffers.

A final consideration of importance involves carbon dioxide effects. At room temperature and high pH, atmospheric carbon dioxide dissolves rapidly in the titration system and markedly alters the amount of titrating

agent required. In a similar way the titration of carbonates and bicarbonates with acid is affected by dissolved gas set free during the reaction. Carbon dioxide errors are commonly avoided by working with boiling solutions for titrations at high pH. The titration of carbonates may be done accurately by the selection of an indicator which changes at low pH and thus compensates for the carbonic acid or by boiling the carbon dioxide from an acid solution and subsequently adjusting to a neutral endpoint. In the latter case an excess of known acid is added, and after boiling the system is brought back to the endpoint with an alkali solution of known strength.

### *Oxidation-Reduction Indicators*

In many respects oxidation-reduction indicators are analogous to the pH indicators. Two forms possessing different colors must exist in both cases, and the conversion of one form into the other must be reversible. The reaction of the oxidation-reduction indicator may be represented as:



where  $I_{red.}$  and  $I_{ox.}$  represent the reduced and oxidized indicators respectively and  $ne$  the number of electrons withdrawn when the reduced form is oxidized.

The iodine-iodide system supplies an excellent example of an oxidation-reduction indicator. When iodine is added to a reducing agent like thio-sulfate or ascorbic acid, a colorless system is obtained and no color change occurs as long as reducing agent is present. However, when the first excess drop of an oxidizing agent is added, a small amount of iodine is formed, turning the solution yellow. If starch is included in the system a much more striking color change is observed, from colorless to an intense blue. It will be obvious that iodine itself may often be used to advantage as both the indicator and the oxidizing agent. A survey of common practice reveals that the indicators of this class are used ordinarily with rather strong oxidizing and reducing agents.

Certain oxidation-reduction indicators react slowly and care must be observed to avoid overrunning the endpoint. The addition of catalysts when known is then a useful adjunct. Occasional incompatibilities are encountered with these indicators involving the formation of an insoluble material through which the indicator is lost.

### *Indicators for Special Ions*

Indicators which reveal the presence of small concentrations of particular ions are useful, especially in the volumetric determination of halides and silver. One type depends on the change of a colorless ion into a colored



precipitate. In a method for the determination of chloride by titration with silver nitrate, a little chromate ion is added. The extremely insoluble silver chloride is formed and separates until the chloride ion concentration is very low. A slight excess of silver ion then will form the slightly soluble silver chromate which is red. Appearance of this red color is taken as the endpoint. Since a trace of chloride still remains and some silver is consumed in the reaction with the chromate, a small correction becomes desirable with dilute solutions (0.01 N). The quantitative aspects can be calculated readily from solubility product data and errors may be anticipated by such means. In principle the process is actually a quantitative fractional precipitation and is treated accordingly.

If a very small amount of fluorescein is added to a chloride solution, a faint yellow-green color results. When silver nitrate is added, colloidal silver chloride is obtained which subsequently flocculates near the equivalence point. At the completion of the reaction the precipitate suddenly turns red. The fluorescein in such a system is known as an adsorption indicator, and its action has been explained in the following way.

A precipitate shows a tendency to adsorb ions of the kind making up the precipitate. Silver chloride suspended in sodium chloride preferentially adsorbs chloride ions, and the suspended particles become negatively charged. Fluorescein is a weak acid and also supplies anions but these are not strongly adsorbed, and the color due to fluorescein therefore remains in solution and is unchanged. However in silver nitrate solution a precipitate of silver chloride strongly adsorbs silver ions, and the particles acquire a positive charge. Therefore, if one titrates a halide or thiocyanate with silver ion, the particles of precipitate abruptly lose their negative charge and take on a positive charge at the end point. The adsorbed excess of silver ions then reacts with the anion of the indicator to form a colored compound, and the precipitate suddenly takes on that color. In such procedures the indicating material is known as an *adsorption indicator*.

#### THE NATURE OF THE STAINING PROCESSES

Colored compounds owing their color to the phenomena discussed in the preceding sections are used in staining. Two general properties are necessary before any substance is useful as a stain. The substance must be colored or react in the system to give a colored product, and some portion of the system must preferentially retain color while other portions do not. These two conditions are imposed of course because the aim is to increase the color contrast in the object system. In bacteriology this aim is ordinarily accomplished by a direct staining of the organism while the background is left uncolored. Sometimes it is convenient to use a *negative* or *background* staining process which dyes the background and leaves the organisms unaltered.

In addition to rendering the entire organism more visible, dyes have been employed to:

- 1) Display the structure and finer details of bacteria.
- 2) Reveal the distribution and chemical nature of cell constituents.
- 3) Determine pH and oxidation-reduction potentials both extra- and intracellularly.
- 4) Differentiate between organisms.
- 5) Distinguish dead from living cells.

Before considering these applications it is desirable to review theories of staining.

The problem of the mechanisms of staining was much discussed in the past, and a sharp difference of opinion arose. One school of thought maintained that staining and dyeing were the results of physical forces, and the other school held that only chemical reactions were involved. With the advance in knowledge of the molecular basis for both chemical reactions and such physical phenomena as solution, absorption, adsorption, etc., it has become a shibboleth of the textbook writer to state that there is really no meaning in the controversies over the physical or chemical nature of many phenomena, staining included, and to leave the subject. But this is an intellectually poverty-stricken solution of a real problem which has productively agitated debate and stimulated research. Such treatment amounts to a refusal to acknowledge the existence of perfectly well-known natural variations that may properly be labeled physical and chemical processes. It may frequently happen that problems are beyond us at any given time, and that research cannot be effectively done until the necessary related fundamentals have been worked out. Yet to ignore the very existence of these problems can only hinder their ultimate solution.

Most experimentalists, of course, recognize the need for an understanding of the staining mechanism, but active research has been hindered for two reasons. The substrates in staining procedures are known to be exceedingly complex, and to date this complexity appears increasingly greater as more is learned. Work is therefore concentrated more upon the components of tissues and cells since a basic knowledge of these is required before much can be learned about the manner in which they are dyed. Secondly, investigators are waiting upon the discovery of entirely new approaches to the study of the forces responsible for staining. It has become increasingly evident that both the physical and chemical theories of staining are useful in particular situations, but that neither alone accounts for all of the observations in all of the cases.

It is desirable to begin by giving some thought to the characteristics of physical and chemical processes. At the outset let us recognize that forces



are responsible for both types of processes and that these forces are frequently electrical in origin when one is considering phenomena at the molecular level. These electrical forces arise from the outer electrons of the participating atoms and apparently vary in magnitude whether they differ in kind or not.

#### THE NATURE OF CHEMICAL PROCESSES

The classical chemical processes involve the forces responsible for the formation of the valence bonds. Formation of such a bond results from the transfer of electrons from atom to atom or from the sharing of pairs of electrons by two atoms. In the first case (the *ionic bond*) discrete charges appear that are fixed to a particular atom or group of atoms. These charges are small integral multiples of the charge on the electron. Pairs of electrons when shared by two atoms may both be supplied by one atom as in *coordinate bonds* or one may be supplied by each atom as in *covalent bonds*. These three kinds of valence bonds lead to the formation of chemical compounds and often, though not always, involve rather large energies relative to the physical processes. The chemical processes in general result in the formation of new substances which are subject to isolation and description.

#### THE NATURE OF PHYSICAL PROCESSES

On the other hand, typical physical processes never lead to new compounds but deal instead with diffusion, changes in state, solution, and interfacial tension. The force responsible for diffusion arises from the thermal kinetic energy of the molecules and leads to dispersion of released gases, etc. It is known to be due to the movement of the molecules which increases as the kinetic energy increases.

After a compound is formed by chemical reaction there are forces remaining which tend to cause aggregation of the molecules. These forces are sometimes called *residual*, *secondary*, or *van der Waals* forces. They may be illustrated by means of a typical covalent organic compound like benzene. At 85°C. the force arising from kinetic motion and tending to disperse the individual molecules is greater than the secondary forces tending to hold the molecules together, and benzene exists as a gas. To be sure there is still a limited molecular interaction, and the ideal gas law, assuming the complete independence of the molecules, does not hold until the temperature is raised still higher. When the dispersing force is decreased by lowering the temperature, the force of aggregation remains essentially unchanged but gains in relative importance. Below the boiling point (80°C.) liquid exists with a density much greater than that of the gas phase. The molecules still



have kinetic energy and move, but the mean free path is now shorter. In other words the average distance between molecular collisions is small, and each molecule is always under the influence of many neighboring molecules.

In the solid state the molecules have almost ceased to wander about, and the forces of aggregation are more important than ever. The individual molecule still has kinetic energy, but it is manifested mainly as a vibration about an average position in the crystal lattice. Some wandering does apparently occur but it is very small, and the substance is described as a rigid solid.

Formation of a solution is somewhat like vaporization. The forces of aggregation in the solute are overcome by the forces of dispersion exerted by the solvent phase, and the solute becomes uniformly distributed throughout the solvent by the process of diffusion.

If the solvent is a liquid, for example, surface anomalies exist. Surface tension is a resultant force acting on the surface molecules and is slightly in excess of the forces in the interior of the liquid. This excess force apparently arises in the surface because the outer layer of molecules cannot satisfy its normal force of aggregation on a still higher level or superimposed layer of liquid molecules. It would appear that this force reacts instead with neighboring molecules in the surface, and all are thus more strongly attracted together in a layer than neighboring molecules are attracted throughout the body of the liquid. In solutions the surface layer will contain either higher or lower concentrations of solute than does the solution proper. The exact relationship will depend upon the nature and magnitude of the residual forces of both the solvent and the solute molecules.

#### DIFFERENTIATION OF PHYSICAL AND CHEMICAL PROCESSES

Typical physical processes are usually easy to distinguish from typical chemical reactions although complications do arise. An example of a difficulty might be found in the condensation of individual acetic acid molecules from the gas phase to liquid acetic acid. In addition to the physical process of liquefaction the molecules combine to form dimers (pairs) whose properties differ slightly from those expected for the monomeric acetic acid. Thus a new substance forms, and a chemical process has taken place. It is true that the change of physical state is abrupt while the dimerization reaction is largely complete in the gaseous acid and increases as the temperature drops. However, two such processes may overlap and affect each other somewhat thus making it difficult to consider either one separately.

A still more complex situation arises when two or more phases are present, especially when one is a solid. Interfacial tensions, similar to those of gas-liquid surfaces, exist and produce great changes in the local concentrations of solutes. If a solute concentrates at the surface of a solid phase in contact

with a solution it is said to be *adsorbed*. When the solute is dispersed throughout the solid, the term *absorbed* is used. Since it is often difficult to determine whether the solute is dispersed throughout or concentrated on the surface of a finely divided solid, the term *sorbed* (*sorption*) is coming into common use to signify an indefinite case.

Ordinarily it is extremely difficult to decide whether the solute is held on a solid phase by the secondary valence forces of aggregation or by the chemical forces of ionic, covalent, or coordinate bond formation. It has been customary in the past to consider adsorption as a physical process, and in general this assumption may be valid. However, there is now evidence of the formation of unstable but definite compounds when some solutes are taken up by some solids. Most such observations stem from the work with catalysts which at least occasionally form compounds with one of the reactants in the catalytic system.

#### MECHANISMS OF STAINING AND DYEING

It is plain from the foregoing that the mechanisms of staining offer difficult problems for the research worker. For if chemical compounds are formed, their isolation and characterization would be exceedingly troublesome since so little is known of the complex substrates encountered in biological work. Nevertheless, many attempts at elucidating staining mechanisms have been made, and a portion of the findings will be discussed below.

#### *Staining by Physical Processes*

Physical forces are unquestionably responsible for the meeting of the dye and substrate since for this process there is no need to assume changes other than solution and diffusion. This statement will apply even in the event that the substrate has a typical chemical affinity for the dye. In some of the apparently simple cases, physical changes seem completely adequate to account for the actual staining process. A discussion of a few such instances follows.

The action of fat dyes such as those of the Sudan series in the staining of lipid substrates can easily be explained in terms of ordinary solubility properties. These dyes contain no strongly polar groups and would be expected to dissolve in ordinary nonpolar materials of the fat and "fat soluble" types. On the other hand the solubility of Sudan stains in polar substrates, carbohydrates, and aqueous phases is very low. Obviously when the dye solvent and substrate are not completely miscible a partition equilibrium will be established which is dependent upon the natures of the substrate, dye, and dye solvent. In general if the similarity in polarity between the dye and substrate is greater than between the dye and its solvent, the dye



concentration will be greatest in the substrate. The Sudan dyes for lipid substrates therefore can be best applied in water-alcohol solution using the minimum quantity of alcohol required to get the dye into solution. The water, in addition to being a poor solvent, may be important in transferring the dye through outer water-containing structures of the cell.

Another possibility involving true solution might be the formation of *solid solutions*. This type of system depends upon the uniform dispersion at the molecular level of one solid in another. Variation in the properties of the solid components over a broad range does not lead to phase separation. More specifically solid dye will dissolve in the solid substrate, and dye concentration will depend only upon a distribution coefficient just as in the case of the Sudan type of lipid dyes. The extent to which the formation of solid solutions is involved in biological staining is uncertain owing to lack of knowledge of the complex substrates and to the difficulty of demonstrating possible chemical reaction in very small samples of substrate.

Since the substrates of the textile chemist are invariably and those of the biologist are often in the solid state, surface irregularities, minute holes, and the like might well take up dye solutions by capillary action. Presumably some fixation mechanism would be required to hold the dye in position in systems subject to washing after the dye has been applied.

In certain cases staining might be explained by entrapment of crystals of low solubility inside structures from which they cannot escape. This idea may account for staining by compound dyes when the compound dye is formed within the substrate rather than being preformed and then applied. The anchoring effect of mordants may also be due to the formation of insoluble chemical compounds. However, such an explanation probably does not apply in most cases and certainly not for the use of substantive dyes. The textile worker refers to *substantive dyes* as those which will stain fabrics without the application of mordants. A *mordant* is a substance which will anchor or fix a dye to a substrate.

### *Staining by Chemical Processes*

Hypothetical mechanisms of staining may be formulated from a knowledge of the chemistry of dyes and the nature of stainable substrates. There are a number of generalizations that result from experiences in the use of acid and basic dyes which must be explained in any such account. Chief among these are the following facts.

Both the nature of the substrate and pH greatly influence the uptake of dyes. Basic dyes are taken up most effectively in alkaline solutions and least effectively in acid solutions. On the contrary, acid dyes are taken up least strongly in alkaline solutions and most strongly in acid solutions. These are consistent observations indicating the need for knowing and controlling the



pH in staining. The student should recall that with an amphoteric substrate the negative charge will increase with alkalinity, and the positive charge increase with an increase in acidity. Of course, as the alkalinity of a solution of basic dye and the acidity of an acid dye solution are increased there will not always be an indefinite increase in the uptake of dye. For one thing it will be recalled that under extremes of pH there will be formed an increasing quantity of the color base or the color acid of the dye. If these structures are unstable or insoluble, as many of them are, the strength of the dye solution will be decreased and staining intensity actually lost.

In addition to the above observations it is found that strongly adsorbed anions increase and strongly adsorbed cations decrease the amount of a basic dye taken up by a substrate. Uptake of acid dye is favored by strongly adsorbed cations and decreased by strongly adsorbed anions.

From these facts the mechanism of staining would seem to be dependent in part on the electrolytic character of the dye. Staining has therefore been thought of in terms of the reaction of ions with charged surfaces. Whether the forces involved are physical or chemical in nature now becomes a problem of moment. The prominent possibilities are these:

1. The dye is adsorbed and held on the substrate surface by van der Waal's forces, induced dipoles, or hydrogen bonds.

2. Dye is held by coulombic attraction of dye ions to a surface of opposite charge. The charged surfaces of the substrates preferentially attract ions of opposite charge, and a thin layer of relatively high concentration is formed. This mechanism may be involved in the initial part of the dyeing process, but apparently cannot be the only factor ultimately binding the stain to the substrate. If no other binding were involved the presence of excess electrolyte would displace the dye ions from the sphere of influence of the substrate. In general, however, electrolyte increases the dye uptake and tends to reduce the rate at which dye is washed off the substrate.

3. The dye forms a salt type linkage with substrate ions of opposite charge. Any such chemical product must be insoluble to resist washing.

4. Dye reacts with substrate by some covalent linkage to form a new and insoluble compound. In this case it should be possible to formulate the reaction in terms of stoichiometry where the number of reacting groups available to the dye is known.

5. Combinations of these possibilities may occur particularly in the case of chemically heterogeneous substrates.

#### THE MECHANISM OF THE DYEING OF TEXTILES

It will be useful to state briefly a recent concept of the dyeing of textiles. The dyeing of textiles is viewed by Valko as a process of sorption. Under the most effective conditions of dyeing, the dye is dispersed almost com-

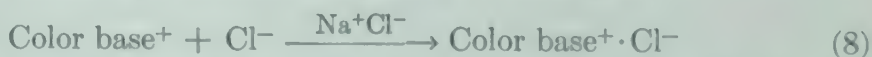
pletely into single ions. The dye penetrates into and is homogeneously distributed throughout the individual fibers of the fabric and is not limited to the external surface of the fiber. The penetration has been demonstrated by sectioning and by X-ray examination of the fibers. The dyeing process is exothermic since the sorption equilibrium is shifted in the direction of decreased dye uptake at increased temperatures. That dyeing of textiles is a process of sorption is also indicated by the accumulation in the fiber of analytical equivalents of counter ions for any given amount of dye taken up. The maintenance of electroneutrality<sup>2</sup> requires this equivalence if the dye ions as such are being sorbed. Thus, dye anions should be accompanied by cations and dye cations by anions.

The quantitative aspects of dye uptake by textiles can be explained in terms of adsorption isotherms and meet the thermodynamic requirements for the *Boltzman law* for the distribution of a compound between two kinds of sites. The Boltzman law is stated as follows:

$$C_D \text{ fiber} = C_D \text{ soln.} \cdot e^{-\frac{W_D}{RT}} \quad (7)$$

where  $C_D$  fiber is the molar concentration of dye ion in the fiber,  $C_D$  soln. the molar concentration of the dye in the bath or vat, and  $W_D$  work gained by transferring one mole of the dye from the fiber into the dye bath.

Most dyes can be caused to aggregate by addition of salts. This can be thought of in terms of a common ion effect, for example,



or in terms of reduction of the repulsive force between the similarly charged dye ions. A substance which destabilizes a dye solution by promoting the formation of micelles or colloidal aggregates of dye will assist the staining process, providing flocculation does not actually become extensive. Contrariwise, a substance which stabilizes a dye by preventing aggregation often restrains the staining process. These two observations were for a long time interpreted as meaning that the effective staining agent was actually a dye micelle rather than the individual molecule.

However, as pointed out previously, there is evidence indicating that the staining of fabrics is due to the individual dye ion. Why then are dyes which tend to aggregate more effective than those which show little aggregation? One satisfactory explanation attributes the tendency toward aggregation

<sup>2</sup> Thus the dyed, individual fibers do not bear a charge. However, if the fiber sorbed only dye ions without the associated counter ions it would acquire an electrical charge. The sign of the charge would be that of the dye ion, and the magnitude would equal the sum of the sorbed ions. As a matter of fact such charges are not observed and would not be expected as long as ions of opposite sign are present.



to strong intermolecular forces. These forces may also allow effective combination of dye and substrate, and the dye is said to be a good dye relative to others showing no tendency to aggregate. On the other hand, if the individual dye ions have already aggregated the intermolecular forces have been largely satisfied, and the colloidal dye has little affinity for the fiber. Furthermore, the large micelle may be unable to penetrate the interstices of the substrate because of its excessive size. In this light the only value of the particles of colloidal dimensions would be as reservoirs supplying monomeric dye to the system by means of a reversible equilibrium.

The role of salts in dyeing seems to be in the reduction of the electrical repulsion between dye ions and similar charges on the substrate. After some dye is taken up the fiber will change in charge unless counter ions are also taken up. Salts increase the concentration of available ions and thereby facilitate the uptake of the counter ions and subsequently of dye ions.

Summarizing briefly, the dyeing of a fiber probably involves surface adsorption as a first and rapid step. The dye then diffuses more slowly into the fiber and is fixed there. The fixation process probably varies from one type of fiber to another. In the ionic animal fibers salt bonds may be formed and coulombic interactions must certainly play a part. Cellulose and nylon do not possess fixed charged groups so that such a process cannot be involved in these fibers. Hydrogen bonding is always possible and undoubtedly helps bind the dye to fiber even in the case of wool and silk. Van der Waal's forces to an unknown extent must also contribute, but the formation of covalent bonds is quite unlikely for stable covalent compounds of dye and fiber are not observed by any method.

Van der Waal's forces seem to fall into the category of the physical, for they do not lead to the formation of new chemical compounds as we usually consider them. However, hydrogen bonds may be important in the chemical structure of a substance like acetic acid and be rather stable bonds. At other times they are quite weak and contribute little to the chemistry of a compound. It is therefore difficult at present to ascertain unequivocally whether the dyeing of a given fiber is a physical or a chemical process or both.

#### THE MECHANISM OF BIOLOGICAL STAINING

Knowledge of the mechanisms of histological staining is not even as far advanced as that of the mechanism of textile dyeing. There are much fewer reliable and quantitative data on staining of cells and tissues available for consideration than for textiles, partly because few quantitative experiments have been attempted. The situation is complicated by the chemical variety of the histological substrates presented to the dye. A cell is infinitely more varied in physical structure and chemistry than any textile. Still there are



no data to support a belief that dramatic peculiarities exist in the case of histological staining. It is therefore reasonable to assume that the mechanisms of histological staining may be similar to those of the dyeing of fibers.

The histological data available suggest that new covalent compounds or insoluble salts are not formed in staining with dye salts. It has not so far been possible to isolate any such new chemical compounds, and if they occurred as a result of the formation of bonds of these types, at least some stable structures would result. Furthermore, the absorption spectrum of the dye in the stained cell is the same as that of the dye in the staining solution. Extensive washing of stained cells will usually remove the dye, and afterwards the cell may be restained indicating that the original substrate has not been removed by the washing process. Therefore, strong chemical bonds are unlikely in the binding of the stain to the substrate. Weaker chemical bonds are not excluded, e.g. hydrogen bonds.

Electrical charges are definitely involved in some staining procedures. Many of the natural substrates are amphoteric in character and consequently have isoelectric points. On the acid side of their isoelectric points such materials are stained with acid dyes. Even nuclear material takes up acid dye when in strongly acid solution although ordinarily only basic dyes are sorbed at normal pH values. In this connection it should be pointed out that a principal component of the nucleus is nucleic acid whose isoelectric pH is very low. Staining above the isoelectric point is best done with basic dyes. These findings suggest that either chemical or physical interaction between charges of opposite sign can be very important in the staining of ionized substrates.

However, some additional factor must be involved even here, for not all basic stains will be taken up in a similar way by a given part of the cell. Therefore, some specificity appears to be required in addition to the interaction of opposite charges. Difficulty in interpretation also arises because staining is usually complicated by development and washing steps in the procedure. Such treatments can greatly alter the picture of staining because of solubility differences existing between the original staining solution and the solvents employed in these steps.

It is commonly observed that substrates do not withdraw all of the dye from a solution. This fact further supports the case against a strong chemical bond but does not allow a decision between the various weaker forces. An equilibrium is reached, but one cannot decide whether hydrogen bonding, dipole-dipole interaction, coulombic charge attraction, van der Waal's forces, or some combination is responsible.

In histological preparations most of the substrates are solids. Many are solid in the natural state, and others are brought into the solid phase by the fixation treatment given the material before staining. The presence of solid

phases provides opportunity for adsorption based completely on physical processes. On the other hand chemical reactions cannot be excluded merely on this basis because many are well known in multiphase systems, for instance the rusting or corrosion of metals, the reaction of carbon dioxide with solid metal oxides, and photographic development.

From this picture one can only conclude that the problem of staining has not been solved and that its solution will require a more extensive knowledge of the substrates as well as the kinds and relative contributions of the forces involved. It will be obvious that there will need to be a vast amount of fundamental work done before any clear cut picture can be drawn. Nevertheless, a difference in response to staining indicates a chemical difference in substrate regardless of the nature of the mechanism. Variations in the chemical composition will produce variations in staining even if a stain is taken up by a purely physical process as in the case of the fat stains.

### FACTORS THAT INFLUENCE STAINING

Methods of staining bacteria and other biological materials generally consist of more than the single step of using a dye solution. The materials to be stained must be properly prepared and then stained in various ways. The usual steps and the factors that influence staining will be discussed individually.

#### *Fixation*

Any process employing either physical (heat or dehydration by freeze drying) or chemical agencies which immobilize the organism and its structures is fixation. Before the bacterial cell is stained it is fixed. Not only does this fixation have the purpose of causing bacteria to adhere to the slide, but it has the equally important purposes of preserving the visible structures and increasing their visibility. Fixation may also be used to change the affinity for dyes as, for example, polyvalent heavy metals may be used to tie up free carboxyl groups while other substances like formaldehyde react with amino groups and thus reduce or increase respectively the affinity for basic dyes. Fixation kills the bacteria, a useful safety measure, and furthermore is necessary since living bacteria are generally impermeable to many of the dyes used.

A good fixation procedure will: 1) avoid shrinkage and swelling of parts of the cell, 2) avoid autolysis, 3) protect cell substances that might otherwise be soluble in the staining reagents, 4) render cell materials more rigid, (This is especially important in fields other than bacteriology where sectioning precedes the staining.) 5) avoid the formation of artifacts. This list represents a formidable group of requirements which is met by no one fixation



procedure in common use. As a result fixatives are chosen to meet the requirements of a given staining procedure or the particular purposes of a study.

The convenient, popular method of heat fixing bacteria to slides is a most uncritical technique. A better procedure is to prepare impression smears by pressing a glass slide or coverslip gently on organisms lying on a solid surface. When the slide is lifted away it will have adherent material which dries rapidly and may then be fixed by any suitable chemical means. A still more satisfactory method for critical cytological work is the scheme of transferring agar blocks to slides for fixation. Pieces of agar with bacteria growing on their surface are cut out and transferred to slides or coverslips, the side with the bacteria being pressed lightly against the glass surface. The slide and agar are then immersed in some chemical fixative. In this procedure the fixative is permitted to diffuse through the agar to the bacteria on the surface of the slide. After a proper interval of time the agar block is gently pushed off the slide with small forceps or a loop. The bacteria will remain behind fixed to the slide. This method permits the fixation of organisms without any prior dehydration.

There is no complete theory of fixation. The common results of the employment of varied fixing agents are the denaturation of proteins and the solidification of cell structure. Yet, in spite of the fact that fixation obviously alters the chemistry of the cell, rather accurate notions can be obtained of much of the structure of the living cell. This observation is not surprising inasmuch as the response to fixation is predetermined by the basic sub-microscopic and molecular structure of the living cell.

A rich armamentarium of fixatives consisting of acids, alcohols, heavy metal salts, and oxidizing agents is available to the histologist for both general and special purposes. The bacteriologist uses all of these and has developed no fixatives restricted in usefulness to the bacteria.

### *The Effect of the Substrate*

The staining properties of organisms must ultimately be traceable to the chemical nature and physical organization of structure. In this regard all of their organic constituents, the lipids, proteins, nucleic acids, and carbohydrates have been implicated as substrates in particular staining procedures. To the amphoteric quality of proteins is usually attributed the characteristic of staining with simple aqueous solutions of acid and basic dyes. At physiological pH values most cellular proteins are on the alkaline side of their isoelectric range, a fact presumably responsible for the greater avidity of cells for basic rather than acid dyes. Depending upon the kind of stains taken up, cells or structures may be spoken of as *basophilic*, taking



up basic dyes; *oxyphilic* or *acidophilic*, taking up acid dyes; or *sudanophilic*, stainable with oil soluble dyes.

The organization and physical dispersion of cellular constituents influence their staining reactions. Thus lipid materials molecularly dispersed in protoplasm, or in combination with protein do not stain with the fat dyes of the Sudan series as they do when present as globules or discrete structures.

The porosity of cellular material undoubtedly influences staining, particularly as regards the length of time necessary for optimum results, although with objects as small as bacteria this factor would appear to be of minor importance. Staining reactions with bacteria can usually be completed in terms of minutes. With larger organisms and tissues it is not unusual to expose specimens for hours or days.

### *Staining Reagents*

These generally consist of simple dyes which are molecularly dispersed in solution. The addition of electrolytes which might cause aggregation must be controlled and in general is avoided. The pH of the staining solution likewise must be controlled and should be known. pH control entails the use of buffers which may have their own specific effects independent of pH. The selectivity of dyes for particular cellular structures may depend upon the pH during staining. For example, acids or low pH buffers may increase the selectivity of basic dye for chromatin. This type of specificity, of course, is probably an expression of the fact that the isoelectric ranges of cell constituents vary, and nucleoproteins or any other substrates with low isoelectric ranges will stain with basic dyes at lower pH values than are found for materials with higher isoelectric ranges.

The nature and quantities of impurities as well as the organic solvents in a dye solution may considerably affect staining either favorably or unfavorably. The most dramatic case in bacteriology of an unfavorable influence is the Ziehl-Neelson acid-fast stain in which excess ionic impurity encountered in commercially available basic fuchsins may lead to the formation of artifacts. Bacilli instead of staining homogeneously are studded with what appear to be globules of dye. This appearance is not related to any known structural feature of the cell.

The staining reagent may be applied for the shortest possible time needed to stain the structure in which one is interested and thus diminish the opportunity for staining extraneous structure. Very often, however, the principle of *overstaining* and *differentiating* is preferred. That is, staining is carried to excess, and then a decolorizing agent or *differentiator* is applied to remove the stain slowly. The structures holding the stain most avidly

will be those to give up their color most slowly. Acids are good differentiators for basic dyes, while alkaline substances serve for acid dyes.

### *Accentuators and Mordants*

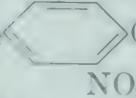
In order to increase the intensity of staining or to have staining occur at all it may be necessary to add an accentuator or intensifier to a staining reagent. At times heat may be used successfully. Chemical accentuators are generally acidic or basic in nature and include such substances as acetic acid, oxalic acid, phenol, potassium hydroxide, soaps, aniline, and formaldehyde. Some of these materials may serve in a dual role as fixatives and as accentuators.

Presumably many intensifiers act by affecting the dissociation of ampholytes present in the cell. Accentuators are to be distinguished from mordants by the fact that they do not act by combining with the dye. Mordants, on the other hand, are chemical substances which have the power of making dyes stain materials otherwise unstainable or increasing their affinity or selectivity for particular structures. Mordants have a strong affinity for both substrate and dye, and in a literal sense they anchor a dye to a substance.

Mordants can be classified as either,

1. Basic mordants which react with color acids. Examples are alum, ferrous sulfate, potassium antimonium tartrate, cetyl pyridinium chloride.
2. Acidic mordants which react with color bases. Examples are tannic and picric acids.

The union of mordant and dye can be demonstrated *in vitro*. Such reaction products are less soluble than the reactants, and the resulting precipitates are termed *lakes*. In the presence of mordants dyes may be *monogenetic*, showing one color, or *polygenetic*, giving various colors with different mordants. Mordants have been used successfully with dyes in all of the possible sequences of application, but varying the sequence may affect the results obtained. The usual practice is to apply the mordant to the substrate first and then the dye. Presumably, after the mordant has united with the substrate it still has unoccupied reactive radicals available for forming salt-like combinations with the dye. In the case of a few mordants such as

picric acid  $\text{NO}_2$    $\text{OH}$ , which has only one ionizing group, this view

seems inapplicable. It is probable that in this case the formation of an insoluble dye-picric acid results in the mechanical entrapment of the lake within the structure of the substrate to which the picric acid was originally sorbed.

## STAINING THE LIVING CELL

Cells may frequently be stained without first killing them. However, since many dyes are highly toxic their application in sufficient concentration will commonly result in death. If the application of the stain kills the cells, this type of staining applied to previously unfixed living cells is called *supravital staining*. The characteristics of the stained cells killed with dye are often significantly different from those of cells dying by natural means or by fixation, thus giving supravital staining a special value in certain studies. One of the useful roles supravital staining can play is as a check or control method against which to compare fixation methods of staining. Distortion of normal structure by fixatives may often be evaluated by comparison against supravital staining. Janus green is one of the most useful supravital stains.

Supravital staining has been used as in the Proca-Kayser method to distinguish dead from living bacteria. Methods of a similar kind have also been suggested for telling dead from living bacterial endospores. Unfortunately, the reports in the literature do not agree as to the true merits of these methods, but the fact that supravital staining is of proven value in other fields of biology would lead one to suspect that potentially these methods can be made to work with bacteria.

Organisms or cells may also be stained vitally, this *vital staining*, also known as *intravital staining*, being defined as the non-toxic coloration of cells. A large literature is available on the uptake of particulate or colloidal dyes by living phagocytic cells. Naturally the bacteriologist is more interested in vital staining by soluble molecularly dispersed dye because large particles of dye do not penetrate the live bacterium. The uses of vital staining have been many, i.e., to stain particular structures within the living cells which ordinarily are poorly visible, to study the permeability of cell membranes, and to determine the pH and oxidation-reduction potential of living protoplasm. The actual realization of these theoretical possibilities is not equally developed and for bacteria in particular is not unequivocally accomplished in any case. Knaysi, who has critically studied vital staining of bacteria, has concluded that any bacterial cell taking dye has lost the power of multiplication. From his results it seems doubtful that any method has as yet been found that gives truly vital staining of bacteria. Of course, if only dead bacteria take up a non-toxic dye one can readily distinguish dead from living bacteria, and Knaysi suggests the application of neutral red for this purpose.

## DETERMINATION OF ISOELECTRIC POINTS OF CELL STRUCTURE BY STAINING

Since acid and basic dyes are known to be taken up in proportion to the number of ionic sites of opposite charge on proteins, the dye uptake can be



used as a rough measure of these ionic groups. Acid dyes will combine with positively charged sites while basic dyes will combine with negatively charged sites, and if the intensity of staining is plotted against pH a sigmoid curve will result for each type of dye. Separate pH curves for the intensity of combination of a given ampholyte with a basic dye and an acid dye should cross where the number of positive charges and negative charges on the ampholyte are equal. Obviously this point of crossing approximately represents the isoelectric point. Whether one accepts a stoichiometric or adsorption theory of staining, the qualitative facts of the application and the theoretical significance of staining at varying pH values is the same. This general procedure has been used to determine the isoelectric points of cellular structures and of bacterial substance.

A critical study of this method has established that the isoelectric point so determined varies with the fixative, the nature and concentration of dyes, and the nature and concentration of the buffers used to control the pH. In general, the staining intensity was found to increase with increasing concentration of dye and to decrease with increasing concentration of buffer. From these results it follows that true isoelectric points are not obtained from the staining procedure, and any values quoted should be correspondingly qualified.

The failure to determine a true isoelectric point by staining at various pH values is not surprising. Protoplasmic structures are chemically complex and are probably composed of numbers of ampholytes rather than a single one. Obviously the experimentally measured isoelectric point of such a structure is a summation of the individual contributions of component proteins and the other ampholytes. This isoelectric point would not be a fixed quantity, and it should shift to different values in the presence of varying kinds and quantities of electrolytes. This shift takes place because the amphoteric components of protoplasm would not all have an identical capacity to react with or adsorb the diverse anions and cations added to the system.

### THE GRAM REACTION

One of the most useful staining procedures in bacteriology is the Gram stain which involves the application of a triphenylmethane dye followed by a mordant containing iodine. Upon the application of a suitable organic solvent, bacteria may be differentiated into a Gram positive group which retains the stain and a Gram negative group which loses the stain to the decolorizer. A number of species exist known as the Gram variables which have an intermediate tendency to lose the dye to the decolorizer.

Originally the Gram reaction was developed as a means for visualizing bacteria in animal tissues, but it was soon realized that a practical tech-

nique had been discovered for the differentiation of bacteria. The response of an organism to the gram stain may be correlated with many other biological characteristics. Table 6 summarizes the available information on

TABLE 6

*The differences between gram positive and gram negative bacteria*

	GRAM POSITIVE	GRAM NEGATIVE
Digestion by gastric juice.....	Resistant	Less resistant ✓
Digestion of killed organisms by trypsin or pepsin.....	Resistant	Not resistant
Resistance to strong alkali (1% KOH).....	Not dissolved	Dissolved ✓
Solubility of lipids in fat solvents...	Resistant	Less resistant
pH of optimum growth.....	Relatively high	At acidic values
Apparent isoelectric point by staining.....	pH 2-3	About 4-5 ✓
Susceptibility to acriflavine dyes....	Marked	Less marked
Bacteriostatic effect of triphenylmethane dyes.....	Very susceptible	More resistant
Inhibition by sodium azide.....	Resistant	Less resistant
Permeability to dye in living state..	More permeable	Less permeable
Bacteriostatic action of iodine.....	More susceptible	Less susceptible
Autolysis.....	Less common	More commonly noted
Lysis by specific complement fixation.....	Not readily apparent	Often observed
Ratio of content of ribonucleic acid to desoxyribosenucleic acid.....	About 8:1	About 1.3:1
Nature of toxins produced.....	Exotoxins	Endotoxins and antigens of Boivin type
✓ Nutrient requirements.....	Complex	Relatively simpler, many species autotrophic ✓
Susceptibility to penicillin.....	Greater	Less
Susceptibility to low surface tension..	Greater	Less
Susceptibility to anionic detergents..	Very susceptible	Much less; susceptible only in acid media
Bacteriostatic action of tellurites...	More resistant	More sensitive
Acid-fastness.....	Some species	None ✓
Rupture by sudden changes in pressure and supersonic energy.....	More resistant	Least resistant

the nature of the known differences that exist between gram positive and negative species. So formidable a list is bound to excite curiosity about the nature of the cellular substrate and the mechanism of the reaction responsible for the gram stain.

Outside of the *Schizomyces* the property of gram positiveness is rare. Ascospores and vegetative cells of yeasts regularly stain gram positive. Some spores and granules in the mycelia of various fungi have been occasionally reported as giving the reaction. The cells of higher plants and of animals are invariably gram negative although the most basophilic structures in these cells, particularly the nucleus and chromosomes, can be shown to lose the triphenylmethane dye-iodine complex at significantly slower rates than other cellular structures. Whether the cause of this phenomenon is related to the mechanism of the gram reaction in bacteria is doubtful, but this subject has not been examined thoroughly. With yeasts it has usually been assumed that the fundamental basis for the gram positive character is the same as that for the bacteria. There are but few data in the literature which suggest this conclusion to be inaccurate.

The great majority of investigators of the nature of the gram reaction have not hesitated to argue theories of the gram reaction from data obtained indiscriminately from both yeasts and bacteria. This procedure raises a question of methodology that concerns bacteriologists in many different circumstances. How many different species should be studied, and what are the criteria to use in picking possible species for studies aimed at establishing generalizations? Individual investigators would probably not be in entire agreement with any detailed answer to this question. Certainly it is not uncommon to proceed on the basis of intuition. But if there is any one sound biological principle that should find general acceptance it is the use of at least two species, each a representative of the taxonomic groups of the widest phylogenetic gap which show the common characteristic. The use of a yeast and a bacterial species to study the gram reaction is consistent with this principle.

The mechanisms suggested for explaining the gram reaction are numerous. To this date it has not been possible to fit together all of the many data which must be assimilated into the framework of a general theory. In solving a complex problem it is often helpful to attempt to separate the problem into its logical constituent parts. Thus the problem of the gram reaction may be thought to consist of not less than five questions: (1) the location in the cell of the substrate of the gram reaction, (2) the nature of the cellular substrate, (3) the physical-chemical mechanism of the reaction of the cellular substrate with the staining reagents, (4) the variations and the causes of these variations in the gram reaction of organisms, and (5) the relation of the cellular substrate of the gram reaction to the properties of the organisms associated with or seemingly coinciding with the gram reaction. Each of these questions will be considered.



*Location of the Substrate of the Gram Reaction*

There is much evidence indicating that the location of the cellular substrate of the gram reaction is in the cell wall. The destruction of the gram positive characteristic of bacteria and yeasts by normal processes of autolysis or by the application of surface active agents or specific enzymes, the nucleases, is often accompanied by the appearance of a granular distribution of gram staining material prior to the total loss of gram positiveness. In these situations one obtains a definite impression of the existence of an outer layer of material which gives a transient "moth-eaten" stained appearance on being attacked by destructive processes. There is available a specific cell wall stain which can be used with the gram stain to determine whether these two different methods stain the same areas of the cell. When a cell stains positive the specific cell wall stain cannot be visibly superimposed. On the other hand, if the gram stain is gradually removed under direct microscopic observation the cell wall staining reagents can be shown to replace it at the periphery of the cell from which the gram stain reagents are being removed.

Stained gram negative cells derived from gram positive cells consistently appear smaller in size than the stained homologous gram positive cells. This observation indicates the presence of gram positive substrate in the outer structure of the cell. The alternative explanation that the gram stain reagents precipitate on the cell surface and thus make the cells appear larger than they really are has been disproved. Measurement of stained gram positive and gram negative cells occurring in the same chains of background stained smear preparations shows the true dimensions of these cells to be the same.

If gram positive material were located in the peripheral structure of the organism it might be expected that in gram negative and positive cells the peripheral area would differ in other respects than in the gram reaction. This expectation has been realized. Gram positive cells treated with tannin retain a secondarily applied basic dye only in an outermost layer while gram negative cells are stained throughout their entire extent. If the basic stain is applied first and then followed by the mordant, only the interior of the gram positive cell stains. By next applying a counterstain the interior retaining the original dye is seen to be surrounded by an outer area which takes the counterstain. On the other hand similarly treated gram negative bacteria are uniformly stained by only the counterstains applied after the mordant.

From the evidence quoted it may be concluded that the cell wall of gram positive bacteria includes material taking the gram stain, but these data do not answer the pertinent question of the exclusive location of the gram

positive staining material in the cell wall. Independent of the gram reaction of the species the cell wall of bacteria is seen as a colored rim surrounding an inner area when stained by specific methods. The evidence for the presence of gram staining substrate in the cell wall has already been quoted. Yet when gram stained cells are observed the entire cell appears solidly colored. From this fact it is not unreasonable to deduce that the gram staining substrate if present in the cell wall is not limited to this structure.

An alternative conclusion might be that in the case of the typical cell wall stain an optical section through a cell having a *transparent* stain in the periphery shows as a colored rim, while with an *opaque* stain in the periphery the object appears solidly colored. These alternative explanations are illustrated in Figure 15. The question of which of these explanations truly ex-



## GRAM STAIN

## CELL WALL STAIN

FIG. 15. Showing the effects of opaque and transparent stains. The gram stain may involve the entire cell more or less uniformly, or it may be confined to a thin shell at the surface of the cell. In either case represented above, the presence of the opaque stain will yield uniformly opaque objects when entire cells are viewed.

The transparent cell wall stain, on the other hand, reveals colored rings when observed in the normal manner. The exact dimensions of the ring and the relative intensity of the stain across it may depend both upon the depth of focus of the objective and upon the plane of focus in the vertical dimension.

plains the difference in distribution of the observed colors can only be settled definitely by future investigation. Inasmuch as there is no proof that the picture observed with the gram stain is due to an opaque color limited to the outer cell structure, the simpler, more reasonable hypothesis is one which regards the stain as being distributed throughout the cell. With yeast ascospores and germinating bacterial endospores it is possible to demonstrate gram positive interiors since in these cases the spore coats do not take the gram stain. In the cytoplasm of yeasts gram staining granules have been repeatedly demonstrated. Therefore, it is necessary to conclude that the gram reaction is not invariably, if ever, limited to materials deposited only in cell walls.

### *Nature of the Cellular Substrate of the Gram Reaction*

The gram stain reaction has been ascribed to a number of substances, lipoproteins and nucleoproteins being most often mentioned by investiga-



tors. Experimental evidence has more and more pointed toward a role for nucleoprotein. Yet there is no known systematic difference in the total nucleic acid content of gram positive and negative bacteria, so that the gram reaction must depend on the presence of specific nucleoprotein rather than on any quantitative difference in total composition.

The rigid structure of the cell wall of bacteria is probably due to the presence of polysaccharide components. In gram positive organisms a magnesium ribonucleoprotein in combination with polysaccharide of the cell wall probably constitutes the primary substrate of the gram reaction. The chief evidence for this point of view is summarized below.

By treatment with bile salts it is possible to extract nucleic acids from gram positive cells with a simultaneous loss of their gram reaction. The resultant gram negative cytoskeletons if kept under reducing conditions by the addition of formaldehyde can be replated with the sodium, calcium or magnesium salts of ribonucleic acid and then will reacquire their gram positive character. The ribonucleic acid employed need not be derived from the homologous species being replated, but desoxyribonucleates, nucleosides, and nucleotides have not been successfully substituted for the ribose type of nucleate. The natural salt of the nucleic acid isolated from gram positive cells is the magnesium salt. Nucleases of various origins, including pure crystalline pancreatic ribonuclease, have been shown to render gram positive cells gram negative. These enzyme studies rather conclusively point to a role for ribonucleic acid in the gram reaction.

Gram positive organisms lose their staining reaction during autolysis. If the process is not permitted to go to the stage of complete disintegration of the cells, gram negative cytoskeletons can be obtained. In the case of *Clostridium welchii* a magnesium ribonucleoprotein has been isolated from filtrates of autolyzed cultures, and this material can be used to replate the gram negative cytoskeletons which then reacquire their gram positiveness. The replating may be separated into two stages. First, the nucleoprotein can be dissociated, the protein moiety used alone, and the magnesium ribonucleate added separately. The gram reaction is not regained by using any other sequence. The specific nature of the protein moiety is indicated by the fact that successful replating depends upon the use of homologous protein.

With purified lysozyme, a carbohydrase, the gram positive reaction can be destroyed. Cells so rendered negative cannot be made gram positive by the application of the specific magnesium ribonucleoprotein. Apparently the replating of gram negative cytoskeletons from gram positive species can only be done when an intact, but as yet unisolated and unknown polysaccharide, remains as a part of the structure of the cytoskeleton or, more probably, the cell wall.

For gram negative species it has not proved possible to alter their stain-



ing reactions by applying any of the materials derived from gram positive species. The difference between these classes may lie in the absence of a specific receptor substance in gram negative species for tying magnesium ribonucleate into the intimate structure of the cell wall.

The magnesium ribonucleate needed to give a gram reaction is only a fraction of the total ribonucleic acid content of gram positive cells as shown in two correlating ways. By shaking gram positive organisms with chloroform large quantities of nucleic acid can be removed without a loss of the gram reaction. Also the confirmatory experiment can be done of removing only about 20 per cent of the ribonucleic acid content of *Clostridium welchii* with a complete loss of the gram reaction. Whether or not nucleic acids associated with the gram reaction differ in composition or structure from those not associated with the staining reaction is a problem as yet unpursued.

The data reviewed prove that the responsible substrate for the gram positive character is a magnesium ribonucleoprotein-carbohydrate molecule or complex. The evidence also indicates that this material is located in the cell wall, but the data do not explain why the gram stain appears to color the whole cell. Consideration of this problem will be included in the following section.

### *Mechanism of the Gram Stain*

Any postulated mechanism of the gram reaction must explain the role of the reagents used in the staining as well as the role of the cellular substrate. There is an extensive literature on the methods of gram staining. From these studies the dyes which have emerged as best for obtaining consistent results are the tetra-, penta-, and hexamethyl pararosaniline dyes, which are found in the commonly used dyes, methyl violet and crystal violet. No adequate substitutes have yet been found.

Iodine is by far the best mordant, the most satisfactory substitutes for it being oxidizing agents capable of forming lakes with crystal violet. Of these the best is mercuric chloride. On adding iodine solutions to the recommended dyes deeply colored lakes of unknown structure form which are highly insoluble in water and only moderately soluble in low molecular weight alcohols and acetone. In the gram reaction the iodine must be applied after the dye, for if the cells are exposed to the mordant and then the dye is applied, decolorization is more nearly the same with both gram negative and positive organisms.

As determined by the uptake of acidic and basic dyes at varying pH the bulk of the cellular substance of gram positive species has its apparent isoelectric point at pH values near 2-3 and gram negative species at about 5. Upon the addition of iodine these values become still more acidic, the

shift being of a greater magnitude in gram positive species and greatest with gram variable organisms such as the *Neisseria*. The reaction responsible for the shift in the measured isoelectric points remains unknown. It has been postulated to be due to a reaction of iodine with lipids although it could involve reactive radicals such as the sulfhydryl groups of proteins. The actual relationship of the isoelectric point of bacterial substance to the gram stain is also unknown. There have been no reports attempting to relate the magnesium ribonucleoprotein-carbohydrate substrate to the observed isoelectric points of the cellular contents. It is necessary to record that the electrophoretic mobility of intact organisms of gram negative and positive species do not differ in any systematic way. Thus if the apparent isoelectric points of the cytoplasmic contents of bacteria vary between gram positive and negative species, this difference is not reflected in an equivalent systematic variation in the zeta potential at the cell surface. Since the magnesium ribonucleoprotein of the gram reaction is a surface component it should contribute to the charge of the cell surface. The studies of electrophoretic mobility, therefore, do not suggest that the substrate of the gram reaction has an unusually low isoelectric point. The differences of apparent isoelectric range of cell substance of gram negative and positive cells then may not involve the magnesium ribonucleoprotein substrate at all, and to this extent the differences in isoelectric range may not be related in any direct way to the gram reaction. This question is, of course, one for future research to resolve.

Numerous organic solvents have been used as decolorizers in the gram stain. All effective decolorizers have the ability to dissolve the dye-iodine lake. Alcohol with iodine dissolved in it will not decolorize cells properly, nor will it dissolve crystal violet-iodine lakes *in vitro*. The best decolorizers for the gram stain act by removing color by solvent properties alone rather than by reason of any acidic or basic properties. In practice ethyl alcohol, acetone, or a 50-50 mixture of the two are effective decolorizers. The difference between stained gram positive and negative species in their ability to be decolorized is relative rather than absolute. In this regard it should be emphasized that the method of fixation markedly influences the capacity of cells to resist the decolorization step.

The gram reaction is shown only by morphologically intact organisms. Bacteria and yeasts broken up by various means, including purely mechanical methods, do not stain gram positive. A most curious fact is the loss of stain to the decolorizer when stained, gram positive cells are broken up. These observations have been used to support permeability theories of the nature of the gram reaction.

Based on the findings discussed, a hypothetical and step by step descriptive picture of the gram stain will now be presented:



1. Application of dye. The basic dye is taken up and diffuses throughout the cell. In general the application of alkali as recommended in various procedures favors the sorption of the dye. The gram positive cells may take up more dye at the pH of staining than the gram negative cells if their cellular substance naturally carries a more intense negative charge. To this extent and independent of any effect of the iodine mordant, gram positive cells are more difficult to decolorize.

2. Application of mordant. The iodine penetrates throughout the cell and reacts to form a water insoluble lake wherever it meets with the previously sorbed dye. As a result the entire cell appears to be colored by the lake. The dye-iodine lake is held most strongly by the magnesium ribonucleoprotein-carbohydrate complex of the cell wall. The organic chemistry of this reaction is entirely unknown and has not been postulated.

3. Application of decolorizer. The decolorizer penetrates the entire cell. In gram negative cells the dye-iodine lake dissolves in the decolorizer and is thus washed out of the cell.

Although any satisfactory explanation of the gram stain would require a knowledge of the reactions between dye, iodine, and substrate, the chemistry of these processes is not known. In spite of this serious deficiency, a great many theories have been proposed for the gram reaction, but in general they are unsatisfactory and incomplete.

Among the several facts that must be included in a valid theory are:

1. There are gram positive and gram negative cells. This difference must be basically due to the fundamental chemistry of the cells concerned. So far the responsible differences have not been clearly ascertained, although efforts are being made in research on the substrate.

2. In addition one must account for the presence of gram negative cells in chains of gram positive cells. Furthermore, the stippled (partly gram positive) organisms must be explained, and in particular the tenacity with which the gram positive spots retain the lake should be considered.

3. The rapid loss of stain to decolorizer when the cells are ruptured is quite striking and is greatly at variance with stain retention by the stippled gram positive organisms. Plainly the integrity of some key part of the cell is destroyed when it is broken open either by the mechanical act of exposing the interior of the cell or as a result of the rapid action of enzymes thus set free. It will be recalled that all cells are presumably fixed and killed at the beginning of the staining procedure, and therefore death *per se* presumably does not control the response to the gram reaction.

4. Finally, the order of addition of the dye and iodine cannot be reversed successfully. This fact is one of the most difficult to fit into a logical theory (and ordinarily can be done so only by assuming special reaction properties for the iodine).



To illustrate the kind of reasoning involved in theories of staining let us develop a hypothesis for the gross mechanism of the gram stain. Assume that the dye is adsorbed by cells in proportion to the net negative charge of the various cellular components. In general this relationship will be true since the positively charged dye cation will be more strongly attracted the greater the negative charge on the proteins, nucleic acids, etc. of the organism. Recalling that the gram positive organisms are made up of materials which have an average apparent isoelectric point two to three pH units lower than those for gram negative types, we would predict that gram positive organisms should have the order of one hundred times greater the affinity for dye than do the gram negatives. The intensity of staining and resistance to decolorization when dye is used without the addition of iodine parallels this expectation.

One may assume that iodine reacts with the dye to form a lake which is somewhat more strongly adsorbed than the dye alone since the lake is not readily withdrawn from gram positive cells. However, the adsorption must not be increased so greatly that gram negative cells hold it with great tenacity.

A serious difficulty arises in accounting for stippled-staining bacteria and the occurrence of gram negative organisms in an otherwise gram positive chain. In the latter case, it becomes necessary to postulate that the scattered gram negative organisms have a high isoelectric point as a result of changes associated with age or resulting from death. Such cells do not appear to be ruptured, but in spite of their appearance they may have invisible breaks in the cell wall and therefore might behave like any broken cell. Consistent with this general pattern the stippled-stained cells must be assumed to possess different isoelectric points in different parts of their anatomy. Unfortunately, there are no data that permit evaluation of this point.

When cells are broken, their pH control mechanisms are lost as a result of the dispersal of the cellular constituents. In this way the decolorizer which has free access to the lake has the opportunity of modifying the pH to a value more suitable for elution of the lake. By this scheme, however, it is difficult to account for the loss of the strong forces that should exist between the lake and materials of very low isoelectric point. To make matters more difficult of comprehension, the use of organic liquids as decolorizers should lower the dielectric constant of the system which ought to have the effect of strengthening adsorption.

The situation is not alleviated by assuming that the lake is specifically adsorbed to a substrate that does not exist in gram negative cells. Such specificity should be retained when cells are ground mechanically because partial fragmentation cannot conceivably break all the adsorption com-

pounds formed, and all must be broken or spotted fragments would be obtained. Nor can enzymic action be blamed since the absence of water throughout the grinding and the decolorization following does not prevent loss of the gram positive character.

In any assumed mechanism the critical order of treatment with reagents poses a difficult problem. One is almost forced to the conclusion that, when applied first, iodine reacts with cellular components so completely that the typical lake is not formed by the addition of dye. On the other hand, when iodine follows dye the reaction between dye and iodine must compete successfully with the reaction between iodine and cellular materials, otherwise no lake would form.

On checking possible hypothetical mechanisms through the list of observations it becomes clear that many important data are lacking and that it is difficult to propose a single complete hypothesis. Perhaps the result to which we give the name gram stain may be achieved by more than one process. At any rate, each proposed mechanism requires so many different specific properties for the system that a critic is led to challenge such mechanisms on the purely statistical basis of it being unlikely that all such unusual conditions can be satisfied.

### *Variations of the Gram Reaction*

Variations in the results of gram staining may have their origin in problems of technic or in changes in the biological substratum of the reaction. The former can be largely controlled by strict attention to the details of procedure and by acting on an intelligent understanding of the conditions affecting the accomplishment of the basic objectives of each of the steps in staining. For example, we should not be surprised to find that the reaction varies if no attempt is made to control the pH at which the dye is applied.

The most critical step in staining is decolorization. The knowledge that water in the organic solvent seriously changes the solubility of the dye-iodine lake warns against the promiscuous application of decolorizer to wet smears and requires information on the water content of the decolorizer. One of the most highly recommended methods is that of Burke (1922) who emphasized this point. As a matter of fact many methods in present day use are but slight modifications of the Burke reagents and procedure.

The gram reaction may vary in numerous ways dependent upon changes in the biological substrate. In many species the gram reaction changes with the age of the culture with young cells more likely to appear gram positive. It has been shown that the resistance of gram stained organisms to decolorization decreases as the culture ages. However, in some species of aerobic spore forming bacilli (*Bacillus subtilis*, *Bacillus vulgaris*) the germinating spore yields a gram negative vegetative cell or a cell with only granules



taking the stain. The organisms may not become positive until several generations after the spore has germinated.

Inasmuch as the fundamental substrate of the reaction appears to be a magnesium ribonucleoprotein-carbohydrate complex any factor which adversely affects the synthesis of this material will tend to give gram negative organisms. Thus organisms grown in magnesium deficient media are gram negative. The inadvertent inclusion of substances in the media or the production by the organism of enzymes capable of attacking the gram positive complex might also be expected to destroy the gram positive character. Similarly, treatment of cells for any reason by chemical or other means that would destroy or modify the substrate would also result in a loss of the gram reaction.

In order that particular cases of variation in the gram reaction may be traced to changes in the magnesium ribonucleoprotein-carbohydrate material there is a need for the study of the conditions of growth and storage that affect the production and the stability of this material. From these studies one might also expect to learn precisely how the gram variable group of bacteria differ from those species consistently giving a definite gram reaction.

In the absence of an external supply of oxidizable material, endogenously respiring organisms lose their gram positive character. For *Bacillus cereus* the interesting observation has been made that endogenous respiration occurs in stages of varying respiratory quotient. The gram reaction is not lost until the respiratory quotient drops to about 0.7. This could be interpreted as meaning that the respiration of the gram stain substrate involves the oxidation of lipid. While these data do not constitute evidence for the nucleoprotein rather than the lipoprotein nature of the gram stain substrate, there is no way of knowing whether the respiratory quotient represents the actual oxidation of the gram positive substrate.

#### *Relation of the Cellular Substrate to Biological Properties Correlated with the Gram Reaction*

The problem of the relation of the magnesium ribonucleoprotein-carbohydrate complex to the biological properties of gram positive cells is an intriguing one, but one on which no work has been done. It has been found that gram positive bacteria in general have different permeability properties than gram negative species with respect to the ability of free amino acids to pass the cell boundaries. This observation certainly is proof of a difference in the structure of the outer layers of gram positive and negative species. The role, if any, of the magnesium ribonucleoprotein-carbohydrate in this difference of permeability remains to be determined.



## THE ACID-FAST STAIN

In 1882 Ehrlich noted that the application of strong acids did not result in decolorization when tubercle bacilli, in contrast to other organisms, were stained with a solution of gentian violet in water saturated with aniline. The tubercle bacilli were thus said to be acid-fast. Acid-fastness has proven to be one of the most useful and significant of characteristics for separating tubercle bacilli and related organisms from all other types of bacteria.

In the Ziehl-Neelsen procedure used nowadays the staining reagent originally used by Ehrlich has been replaced by carbol-fuchsin, a solution of basic fuchsin in aqueous five per cent phenol. The decolorizers used are one to five per cent solutions of hydrochloric or nitric acid in 95 per cent ethyl alcohol. In one important modification of the acid-fast stain auramin is employed. On exposure to ultraviolet light auramin, a yellow, fluorescent diphenylmethane dye, fluoresces in the visible region. Acid-fast cells treated with this dye and an acid decolorizer will fluoresce when illuminated by ultraviolet light while nonacid-fast cells do not retain the dye and do not fluoresce. Microscopes especially adapted for this method of distinguishing acid-fast bacilli use ultraviolet light for illumination and contain yellow filters to remove any blue light entering the ocular. As a result the field of view is dark except for objects containing the fluorescing dye.

The property of acid-fastness is primarily limited to the genus *Mycobacterium*. In addition some few species of acid-fast actinomycetes have been described, and it has been claimed that under proper conditions of growth some species of *Corynebacterium* will appear acid-fast. It is interesting that the few species other than mycobacteria which have been described as acid-fast should prove to be actinomycetes and diphtheroids, organisms which, on grounds other than acid-fastness, have often been considered to be phylogenetically related to the mycobacteria.

Bacterial endospores, ascospores of certain yeasts, the exoskeleton of insects, inclusions in lungs from cases of lipid pneumonia, and ceroids in livers of rats on choline, vitamin E, and protein deficient diets are other materials that have been described as staining acid-fast. The chemical substrates responsible for the acid-fastness of these objects are not similar to those thought to be involved in the mycobacteria. The ceroids seem to have their origin in the oxidation of unsaturated fatty acids. Both by *in vitro* and *in vivo* experiments it has been possible to get oxidation products of unsaturated fatty acids to stain acid-fast.

The mycobacteria are characterized by their unique possession of certain kinds of fatty acids, higher alcohols, and carbohydrates. Among these is mycolic acid, the one compound so far isolated from these organisms which shows the property of acid-fastness *in vitro*. The mycolic acids of different acid-fast species are not identical. All are optically active high

molecular weight hydroxy acids which contain carboxyl and methoxyl groups. The free carboxyl group is necessary for acid-fastness. Mycolic acids are isolated from the unsaponifiable fraction of the chloroform extracts of acid-fast organisms. Upon decomposition under reduced pressure at about 325°C. they yield hexacosanic acid and a colorless non-volatile residue. In the cells the mycolic acids do not exist in the free state but are probably bound in a complex with carbohydrate. The primary substrate responsible for the acid-fastness of mycobacteria is generally thought to be the complexes in the cytoplasm that contain mycolic acid. In favor of this view is the finding that lipid extraction will not result in the loss of acid-fastness until the firmly bound lipids containing mycolic acid are removed. *In vitro* addition of mycolic acid to nonacid-fast substances can result in acid-fastness. There are no cytological data available on the location and distribution of the mycolic acid within the acid-fast cell.

#### THEORY OF THE ACID-FAST STAIN

The demonstration of acid-fastness requires the use of a phenylmethane dye in either aniline or phenol solution. Of all the dyes, basic fuchsin, a mixture of the triphenylmethane dyes rosanilin and pararosanilin, is by far the best. Aniline and phenol may be interchanged, but one or the other of these must be used in the staining reagent. Explanations of the acid-fast stain must assign a role to these reagents.

Phenol and basic fuchsin have been thought to form addition compounds. However, the expressed notion that in acid-fast staining the dye taken up is actually such an addition compound is open to question since in practice one cannot use these compounds to replace the carbol-fuchsin. Nor has it been shown that similar types of compounds form between basic fuchsin and aniline, crystal violet and aniline, malachite green and phenol, or auramin and phenol, combinations which have been used successfully in various acid-fast staining procedures.

The property of acid-fastness is always one of degree. Among both the so-called nonacid-fast species as well as acid-fast species there are actually great differences in resistance to decolorization by different concentrations of acid or in the temperature and time of complete decolorization with a given concentration of acid or neutral alcoholic solvent. It is necessary for any theory to explain these facts as well as why chemically diverse biological materials exhibit the property of acid-fastness. Any theory that cannot be extended to cover all these cases is unsuitable unless one is willing to postulate separate theories for acid-fast bacilli, bacterial endospores, ceroids, etc. A theory of general application would seem to have a greater logical value than one restricted to particular acid-fast objects.

The integrity of the cell boundaries must be maintained if organisms are



to exhibit their acid-fast property, since rupture of the cell by mechanical means and by autolysis have both been described as resulting in the loss of acid-fastness. As a matter of fact the mere mixing and spreading of a suspension of cells on a slide may be sufficient to damage the cell surface and destroy acid-fastness. From these observations the conclusion is inescapable that the cell wall of the bacteria has an important role. Yet the stain appears to color only the interior of the cell and not the cell wall (see fig. 16). One hypothesis offered to explain this distribution of stain postulates the existence of a semi-permeable membrane which allows the basic fuchsin to diffuse into the cell, but on application of the acid decolori-

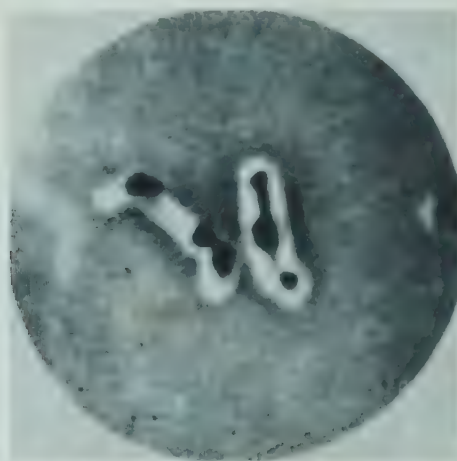


FIG. 16. *Mycobacterium tuberculosis* showing location of beads produced in acid fast staining by the Ziehl Neelsen procedure to be within the organism. Negative staining with nigrosine has been done to outline the outer limits of the organism.

(From Yegian and Vanderlinde, 1947)

zer the basic fuchsin is converted to an "acid" fuchsin<sup>2</sup> to which the cell boundary of acid-fast cells is impermeable. The proponents of this hypothesis claim to show this behavior in model experiments with solutions of dye inside cellophane bags. The difficulty with the hypothesis is the lack of any proof of the conversion of the basic fuchsin to an "acid" fuchsin within the cell. In addition the hypothesis leaves unexplained the association of the property of alcohol fastness with acid-fastness. Washing carbol-fuchsin stained bacilli with neutral alcohol will usually decolorize all organisms but the acid-fast ones.

Acid-fast mycobacteria may appear either homogeneously colored or beaded, that is, colored only in spots. The exact result obtained on staining is dependent upon the nature of the dye reagents used. It is found that the

<sup>2</sup> Chemistry of this change is not stated in the original publication (Sordelli and Arena, 1934) thus the quotation marks are used.



use of carbol-fuchsin, so prepared as to include materials or concentrations of materials that decrease the solubility of the phenol, is most likely to give the beaded appearance with acid-fast cells. The beading is apparently an artifact of staining, for there is no evidence that the beads are dye material collected about or within particular cellular structures. The deliberate addition of electrolytes to carbol-fuchsin, the presence of electrolytes as impurities in the dye, or concentrations of dye which reduce the solubility of phenol in water all lead to beading. In this regard the practical suggestion has been made that if these artifacts of staining are to be avoided carbol-fuchsin should be made up with highly purified dyes and with the hydrochloride rather than the acetate salt of basic fuchsin in a concentration not exceeding 0.3 per cent.

A simple qualitative analysis of the solubility of phenol in water as influenced by the presence of other substances such as basic fuchsin, inorganic salts, and ethyl alcohol is provided by the determination of the consolute or critical solution temperature in the presence of these substances. The *consolute temperature* is the temperature at which the meniscus between two liquids disappears or the minimum temperature at which two liquids become completely miscible. Some such data are recorded in Table 7.

As indicated by the rise of the consolute temperature, these data show that sodium chloride decreases the solubility of phenol in the aqueous phase. However, the effect of basic fuchsin is more complex since a small amount causes a lowering of the consolute temperature whereas larger quantities cause a rise. The exact concentration of dye at which this reversal takes place varies for each batch of commercial dye. By adding sufficient dye the solubility of phenol in water can be decreased to less than is normal for a mixture of phenol and water alone. The addition of sodium chloride, which is soluble in water and not in phenol, then accentuates this loss of solubility by phenol. It is also possible to "salt out" the dye from the water phase by adding high enough concentrations of sodium chloride. The colloidal suspension that is noted for some batches of carbol-fuchsin and for all batches to which inorganic electrolytes are added is the result of a separation of some of the phenol present as a distinct phase. When such a solution is centrifuged two layers are isolated, the more intensely colored and denser phenol layer collecting at the bottom. To sum up, the solubility of phenol in carbol-fuchsin would seem to depend on the relative concentrations of inorganic salts, alcohol, dye, and the nature of the particular batch of dye, and it is those mixtures which decrease the solubility of phenol in water that lead to the occurrence of staining artifacts in the Ziehl-Neelsen procedure.

Beading artifacts appear in the step of the Ziehl-Neelsen procedure in which the stained smears are washed with water where application of water

TABLE 7

*Consolute temperature of phenol-water in the presence of components of carbol-fuchsin*

ITEMS ADDED	CONSOLUTE TEMPERATURE (°C.)
None (control: phenol-water).....	68
Ethyl alcohol, 10 per cent.....	52
NaCl, 0.5 per cent.....	76
Ethyl alcohol, 10 per cent, and NaCl, 0.5 per cent.....	58
Coleman and Bell Co.:	
Basic fuchsin, CF-25, 0.5 per cent.....	54
Basic fuchsin 390547, 0.5 per cent.....	56
Basic fuchsin 390547, 0.5 per cent and NaCl, 0.5 per cent.....	77
Allied Chemical and Dye Corp.:	
<i>Para</i> -rosaniline base, 9753, 0.5 per cent.....	82
Basic fuchsin, NF 40, 0.5 per cent.....	60
Basic fuchsin, NF 45, 0.5 per cent.....	61
Basic fuchsin, NF 43, 0.5 per cent.....	63
Basic fuchsin, NF 43, 1.0 per cent.....	63
Basic fuchsin, NF 43, 1.0 per cent, and NaCl, 0.5 per cent...	78
Basic fuchsin, NF 43, 2.0 per cent.....	74
Basic fuchsin, NF 43, 2.0 per cent, and NaCl, 0.5 per cent...	84
Difco bacto basic fuchsin, 370921	
0.5 per cent.....	53
0.5 per cent, and NaCl, 0.5 per cent.....	78
2.0 per cent.....	42
2.0 per cent, and NaCl, 0.5 per cent.....	79
6.0 per cent.....	52
6.0 per cent, and NaCl, 0.5 per cent.....	88-89

(From Lamanna, 1946.)

If to a mixture of two liquids a foreign substance is added which is only soluble in one of the liquids, their mutual solubility is decreased. In such cases the added substance will raise the consolute temperature. Since they are generally insoluble in organic solvents, salts will usually cause a rise in the consolute temperatures of mixtures of water and organic solvents. The quantitative relation existing between the solubility of an organic solvent in water and in a salt solution is

$\log \frac{s}{s_0} = kc$ , where  $s$  is the solubility of the organic substance in the salt solution,  $s_0$

the solubility in pure water,  $c$  the concentration of salt solution, and  $k$  a constant for a given salt. If a solute soluble in both components of a liquid mixture is added the mutual solubilities of the liquids are increased, and the consolute temperature is decreased. However, when solute is added beyond the point of its saturation in the liquid mixture the mixture will separate into two phases, and the consolute temperature will begin to rise. The separation of the liquids and the rise in consolute temperature will continue to the point of saturation of both phases by the added solute. This situation is observed for basic fuchsin upon its addition to an aqueous solution of phenol as shown by the data of the table.



to the homogeneously stained organism suddenly causes it to appear beaded. Beading is independent of the species of mycobacteria but is dependent upon the employment of a suitable batch of carbol-fuchsin and occurs regularly when sodium chloride is added to the staining solution. Sometimes to get the beads to form it is necessary to tap or vibrate the smear covered with water. It is possible to explain the formation of the beads by postulating that phenol and dye exist in the stained cell as a liquid phase. On adding water to the cell the contact of water with the relatively water insoluble phenol-dye phase results in the occurrence of droplets or beads because the water entering the cell is a material of high surface tension. The beads will later coalesce on the subsequent application of alcohol or phenol solutions because of the solubility of these solvents in the phenol-dye liquid and the consequent reduction of interfacial tension at the surface of the droplets. In this way the effect of tapping slides on the formation of beads, their instantaneous appearance on the application of water, their spherical shape, and their fusion on addition of phenol-soluble solvents can all be accounted for.

This explanation presumes that the use of carbol-fuchsin in which phenol is present to the extent of saturation or supersaturation with respect to water permits the accumulation of phenol within the cell in a similar state. The presence in the cell of water insoluble lipids and of salts insoluble in phenol would lower the solubility of phenol in water. The more salt in the staining reagent the greater the salting out of the dye from the water into the phenol entering the cell. The more dye accompanying the phenol the more likely the resulting solution in the cell will have a reduced solubility in water.

The above explanation of beading is further supported by evidence that the phenomenon is not limited to acid-fast organisms. Four unlike nonacid-fast-bacterial species have been studied; *Bacillus megaterium*, *Corynebacterium diphtheriae*, *Corynebacterium hofmanni*, and an unidentified vibrio. Cells of these species when stained with carbol-fuchsin are rapidly decolorized by alcohol. But when washed with cold water they are decolorized more slowly and at varying rates. Most important of all is the fact that, with the exception of *Bacillus megaterium*, decolorization with water is preceded by the appearance of highly colored beads within the cell when a batch of carbol-fuchsin that contains sodium chloride is used to stain the cells. With *Corynebacterium diphtheriae* decolorization with water was least rapid, and the beads were evident for the greatest length of time before being completely washed out of the cells.

The reason for the attention devoted to the phenomenon of beading is that its analysis has provided a theory of solution as the explanation of



acid-fast staining. This theory may be stated as follows. Cells bathed with carbol-fuchsin take up phenol and dye preferentially from the staining solution. This hypothesis agrees with the fact that carbol-fuchsin stained cells generally appear more intensely colored than the staining reagent applied. In other words the dye and phenol are shared between the carbol-fuchsin and cell according to a distribution coefficient favoring a higher concentration within the cell. This observation is expected since all cells would normally contain materials favoring the greater solubility of the phenol and dye within the cell than in the carbol-fuchsin. Obviously the ever-present cellular lipids, soluble in organic solvents but insoluble in water and including such substances as complexes containing mycolic acid, would play the most important role in determining the distribution of dye and phenol between the cell and decolorizer. The dye-phenol in the cell acts as a part of a liquid phase.

If the cell wall retards the penetration of the staining reagents because of its permeability characteristics, as in the case of the acid-fast organisms and bacterial endospores, the temperature must be raised to accomplish staining within a reasonable length of time. On applying a decolorizer to nonacid-fast cells, the dye and phenol leave the cell because of their greater solubility in the decolorizer than in the cell. With acid-fast cells the solubility of the dye-phenol is postulated to be relatively greater in the cells than in the decolorizer. Thus only small quantities of color would be lost to the decolorizer penetrating into the cell. The cell wall of the acid-fast organism would need the property of resisting the rapid movement out of the cell of any of the dye and phenol that did dissolve in the decolorizer within the cell. It should be obvious that actually the dye-phenol within the cell would not be *absolutely* insoluble in any of the acid and alcohol decolorizers used routinely, but the low order of solubility plus a slow rate of diffusion out of the cell would permit maximal retention of the stain by acid-fast cells. On the other hand rupture of the cell boundaries results in loss of acid-fastness because the stain would now be directly exposed to the large quantities of solvent in the decolorizer bath. The low order of the solubility of the stain in the decolorizer while in the cell would no longer be sufficient to prevent noticeable loss of stain.

These concepts can explain why the differences in acid-fastness are of degree. Since the qualitative and quantitative chemical compositions of cells of different species vary, the actual situation with regard to the relative solubility of the dye-phenol mixture within the cell and decolorizer would be complex and different for each kind of organism.

If phenol and dye are present as such in acid-fast cells and if the staining properties of acid-fast cells stained with carbol-fuchsin are dependent on the relative solubility of the phenol and dye in water and cell constituents

as has been interpreted from the data on the beading phenomenon, then it should be possible to decolorize these stained cells merely by immersing them in water at the consolute temperature. The presence in acid-fast cells of constituents which reduce the solubility of phenol in water would not abolish the consolute temperature, they would only raise it. This theoretical expectation is met. *Mycobacterium tuberculosis* (strain H37) can be decolorized with water at 90°C. while a strain of *Mycobacterium phlei* loses the stain at 80°C. in water and a strain of the avian tuberculosis organism can be decolorized in water at 70–75°C.

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## CHAPTER VI

# The Structure of Eubacteria

### SLIME LAYER

The *slime layer* is an accumulation of viscid material around the external surface of the cell wall of bacteria. It is generally considered to be a non-living secretion or excretion of the organism without an active role in the metabolism of bacteria. No evidence of a disturbance in the metabolism or viability of bacteria appears when the slime layer is removed. These observations are sufficient grounds for not considering the slime layer as living matter and thus as a part of the protoplast. Experimentally it is observed that organisms embedded in their slime layers are frequently more resistant toward desiccation than in the absence of slime layer material. This resistance is probably due to the hygroscopic properties of slime layer materials which may act to hold water in dry atmospheres.

The slime layer material may be either an amorphous accumulation in which the bacteria are embedded, or it may be distinctly oriented or organized about each organism or pair (as in the case of the pneumococci). In the latter situation the slime layer is most often referred to as a *capsule* and on direct microscopic examination may appear as an uncolored "halo" about the cell.

Slime layer material has a viscid jelly-like consistency and in some cases appears quite stringy on manipulation with a loop. These properties are suggestive of a high molecular weight and an asymmetric shape of the molecules composing the substance of the slime layer. The refractive index, not much different from the growth menstrua in which bacteria multiply, makes it difficult to observe the capsules by direct microscopy. The application of simple solutions of acidic and basic dyes is equally unsuccessful in providing contrast since the affinity for dyes is low or entirely absent. Staining procedures for revealing slime layer materials invariably employ mordants. In the electron microscope the slime layer is not sharply defined and may be invisible indicating a low order of opacity for the electron beam.

In spite of the evident similarity in many of the physical properties of slime layer material from different organisms, the chemical composition varies greatly for different species of bacteria and is characteristic for particular species. Not all organisms are known to produce a capsule, nor does any one organism produce slime layer material under all circumstances

of growth. In all the cases so far studied capsular material can be shown to be either antigenic directly or to act like a haptene. There are three major kinds of known slime layer materials:

1. Carbohydrate in nature.

a. Types containing nitrogen. Best known is the polysaccharide of the pneumococcus capsule containing glucosamine.

b. Types not containing nitrogen.

Dextrans. Polymers of glucose with molecular weights of the order of millions. One studied in detail is that from *Leuconostoc mesenteroides*.

Fructosans or levulans. Similar to dextrans except that they are polymers of fructose.

Galactosans. Polymers of galactose.

Cellulose. Found only in species of *Acetobacter*.

Carbohydrates containing hyaluronic acid.

2. Polypeptide. Produced by aerobic spore forming species including the pathogen *Bacillus anthracis*.

The chemical components of the slime layer of a given species at most appear to be few in number or to consist almost entirely of a single type of unit substance, but there may be variations in the nature of the components depending on the circumstances of growth. Some aerobic spore-forming species have been described as producing levulans in sucrose media and a polypeptide type compound under other circumstances. Depending on the organism, the production of a slime layer may or may not be related to the presence of particular substrates in the growth medium. Thus *Streptococcus salivarius* produces a characteristic slime layer containing a dextran only in the presence of sucrose, while virulent pneumococci produce capsules as a normal consequence of growth and independent of the presence of any known specific nutrient. The pneumococcus also illustrates the finding that differences in chemical composition may exist in the slime layer of strains of the same bacterial species. While the pneumococcus strains are looked upon as members of a single species, the different strains of this organism possess antigenically distinguishable polysaccharides. To date more than seventy such antigenic strains have been reported and designated by a system employing Roman numerals.

Of all the chemical constituents of bacterial structure, slime layer material is the most accessible for isolation and study. As a result a considerable literature is available on the nature and structure of slime layer material and especially on the polysaccharides of the pneumococcus capsule. Yet in spite of the great effort expended it cannot be unequivocally concluded that the structure of the material isolated from organisms is identical to the structure as it occurs in the capsule. For example, the isolated type III



pneumococcus polysaccharide on treatment with sodium metaperiodate will react with the Schiff's reagent for aldehydes. On the other hand treatment of the whole cell with periodate does not result in a reaction of the capsule with Schiff's reagent. This observation indicates that the polysaccharide in the organized capsule is substituted on the second or third carbon atoms of its glucose residues and that an attached substituent is released upon separation of the capsular material from the organism.

One of the fundamental questions of biochemistry is the problem of the synthesis of cellular proteins from amino acids. It so happens that the

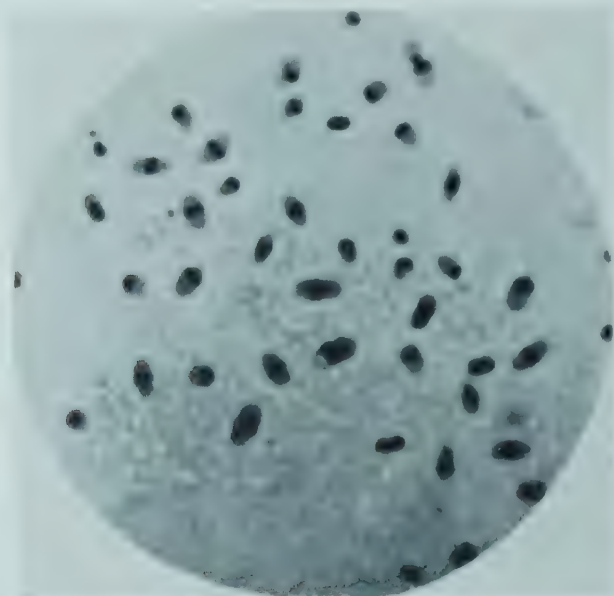


FIG. 17. A unique published example of a transverse capsule. The organism is probably a strain of *Aerobacter aerogenes*.

(From Thompson, 1934)

polypeptide capsular material produced by members of the genus *Bacillus* is composed exclusively of the laevo-rotary, or so-called unnatural, optical isomer of glutamic acid. The synthesis of such a slime layer polypeptide by species of *Bacillus* offers a unique possibility for the study of the general problem of protein synthesis since it provides a system more easily analyzed than the usual obligate intracellular synthesis of protein. In addition there is the advantage of relative simplicity since the synthesis involves only one kind of amino acid in contrast to other protein syntheses which involve the incorporation of a variety of amino acids into single molecules.

The origin of the slime layer is still debated. The thought has been offered that the slime layer is a modification of the cell wall, but there is little evidence for this point of view. The slime layer has also been regarded as a cellular secretion. An organism secreting a viscid material of high molecular

weight that tends to go into solution in the medium only slowly would obviously tend to surround itself with this material. If this concept is correct it is logical to ask whether the secretion by the bacterium is general over its entire surface or limited to particular areas. The general conformation of the shapes of capsules to the shape of the organism would suggest the former to be true. There is in the literature the interesting and exceptional case of a transverse-shaped capsule (fig. 17). Such a case might be explained by secretion at a limited area of the cell about which the major portion of the slime material accumulates and results in the unusual orientation.

The secretion theory raises the problem of how large molecules could diffuse through the cell barriers without resulting in the general diffusion and loss of essential but small cellular substances. A possible explanation is the limitation of synthesis of the slime layer material to a zone adjacent to the external surface of the cell. Extension of this idea leads to a hypothesis suggesting that the capsular material forms in the medium after which it mechanically adheres to the organisms. In support of this concept is the isolation from the sterile culture filtrates of some species of extracellular enzymes capable of the synthesis of slime layer materials in the presence of specific substrates. This finding has been made with *Leuconostoc mesenteroides* which produces a dextran from sucrose. Actually there is, of course, no logical reason why the origin of the slime layer in all organisms should be identical; each organism must be studied. The cases where extracellular mechanisms are or can be found do not exclude the possibility that a secretion mechanism is responsible for the origin of the slime layer with other species and particularly for organisms like the pneumococcus which show a more definite morphological fixity or organization in the appearance of the slime layer.

The possibility of the extracellular synthesis of the capsular substances of high molecular weight has interested students of metabolism. Since the coupling of monosaccharides into polysaccharides is an endothermic process, the energetics of the reactions occurring outside of cells has aroused interest. This problem is considered in the discussion of metabolism.

### THE CELL WALL

The presence of a cell wall as a characteristic feature of the morphology of eubacteria was deduced by the early bacteriologists from the apparent rigidity of these organisms and from their resistance to dissolution by acids and alkalis. The latter property was considered to be analogous to the findings with fungi which have a proven cell wall. The low refractive index of the cell wall made it difficult or impossible to see by either direct or dark field microscopy. In addition, the bacterial cell wall has only a slight affinity



for dyes and was not demonstrable by the early workers with the staining techniques available to them.

The first direct demonstration of the cell wall was accomplished by means of plasmolysis experiments during which the cytoplasmic contents of organisms were retracted from the inner surface of the cell wall which did not collapse. A faintly outlined "ghost" cell that was partially filled by globules of the contracted, more refractive cytoplasm thus was obtained.

The direct demonstration of the cell wall has now been achieved by a number of other means. These include microdissection, differential staining procedures employing mordants, and electron microscopy. From observations by these and still other methods it has been possible to acquire a developing, if still incomplete, picture of the physical structure and chemical nature of the cell wall.

The cell wall accounts for the rigidity, ductility, and elasticity of bacteria by reason of its physical properties. The rigidity of bacteria is indicated by their lack of a spherical shape in an aqueous medium, a shape that would be expected for non-rigid objects subjected to the tremendous surface forces acting on objects as small as bacteria. An interesting demonstration of rigidity has been provided by ultra-centrifugation experiments with *Spirillum volutans* at 400,000 times the force of gravity without any evidence of subsequent distortion in the spiral shape of the organism. Variations in the ease of rupture of bacteria by various means including ultra-sonic vibration reveals differences in rigidity among species. On rupture jagged lines of fracture occur in the cell wall which can be attributed to the rigid character of the structure.

Materials which can be drawn out or hammered thin without fracture are said to be *ductile*, and the cell wall possesses this property. Ductility of the wall has been admirably shown for *Mycobacterium tuberculosis* using preparations of these organisms dried upon collodion membranes. Stretching of the collodion membrane stretched the adhering cell walls of the bacilli without forcing their separation (fig. 18). *Elasticity*, or the ability of a material to recover its size and shape after deformation, is another demonstrated physical property of the bacterial cell wall. Thus bacteria when propelled with force against a solid target will bend but resume their original shape. In addition Wamochser, who succeeded in microdissecting a number of different kinds of bacteria, noted the elastic response of the cell wall to manipulation with microneedles. Elasticity also allows the reversible expansion and contraction of organisms subject to changes in their turgor pressure.

The observed thickness of the cell wall varies with the method used for its measurement. For dried preparations as observed with some staining procedures or with the electron microscope, the dimensions are considerably



less than would appear to be true for the same material observed in wet mounts. *Bacillus cereus* in electron microscope pictures shows a cell wall thickness of about one sixteenth the diameter of the cell. For an organism

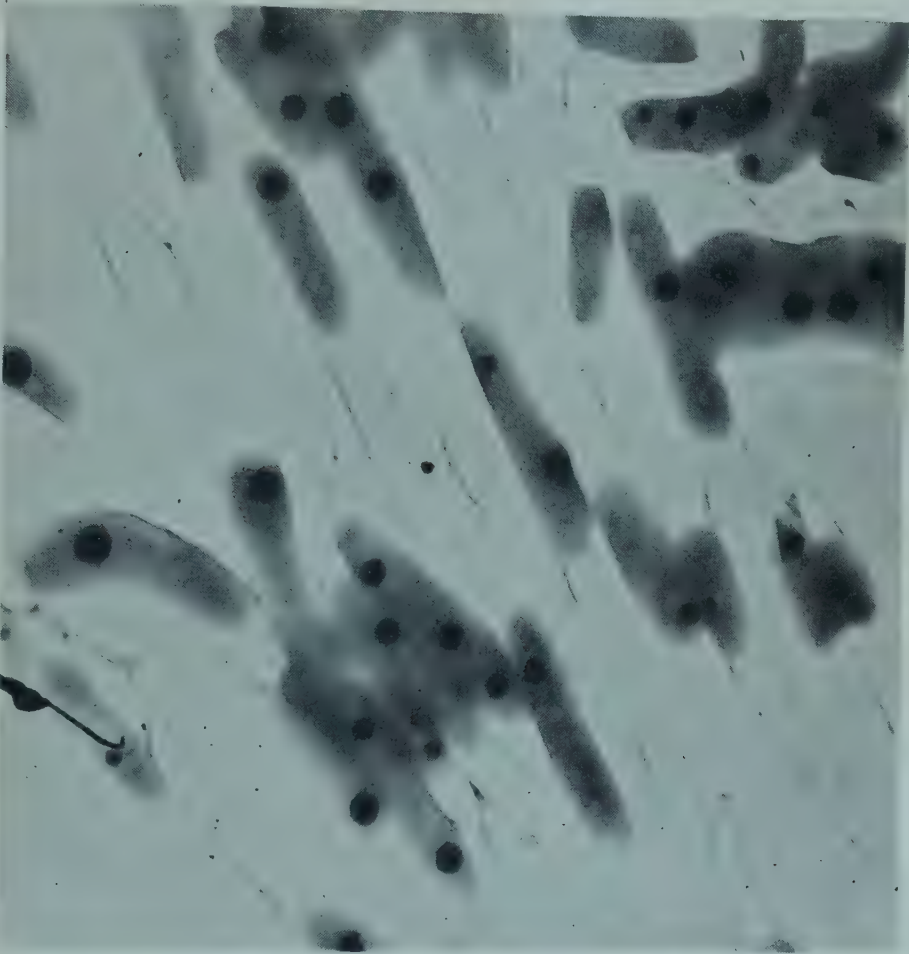


FIG. 18. An example of the ductility of the cell wall in an avian strain of *Mycobacterium tuberculosis*. The cell walls are stretched out and the cytoplasm thus appears to occupy only a portion of the volume occupied by the cell wall. The stretching resulted from shrinkage accompanying the drying of the cells in preparation for taking electron microscope pictures. The above picture was taken with the electron microscope at a magnification of 11,350 $\times$ .

(From Knaysi, Hillier, and Fabricant, 1950)

like *Mycobacterium tuberculosis*, which rarely exceeds a diameter of 0.5  $\mu$ , Knaysi gives the figure of 0.023  $\mu$  as the thickness of the cell wall. In this case about one tenth of the width of the cell is occupied by the total cell wall. From such data it is evident that in spite of the custom of referring to the "thinness" of this structure, the cell wall makes up a considerable proportion of the total volume of the bacterial organism. Assuming a

bacillus to be a cylinder, an organism  $1\ \mu$  in width by  $2\ \mu$  in length with a cell wall  $0.05\ \mu$  thick would have a total volume of  $1.53\ \mu^3$  of which  $0.362\ \mu^3$  or about 23 per cent is occupied by the cell wall. A coccus  $1\ \mu$  in diameter with a cell wall  $0.05\ \mu$  thick would have 25 per cent of its volume occupied by the cell wall (see Table 8).

Knowledge of the chemistry and arrangement of the chemical constituents of the cell wall is poor. Few purified compounds have been isolated from bacteria for which incontestable evidence indicates their origin to be the cell wall. Claims for the presence of cellulose, hemi-cellulose, glycoprotein, and chitin in the cell wall have been made, but these reports are not the result of critical studies by chemically specific and reliable methods. In view of the great progress which has been made in studies of the cell wall

TABLE 8

*Comparison of the composition of bacilli and isolated cell walls of Corynebacterium diphtheriae*

	DRIED BACILLI	CELL WALLS (55.4% BY WEIGHT OF CELLS)
Protein.....	79.56	88.02
Fats.....	3.45	1.44
Water.....		0.91
Carbohydrate.....	5.28	7.94
Ash.....	8.11	1.69

(From A System of Bacteriology, 1: 123.)

of fungi and higher plants it is surprising that equivalent knowledge of the organization of the bacterial cell wall should be as poor as it is. Certainly many of the methods used by plant anatomists and students of physical and textile chemistry should be applicable to studies of bacteria. The necessary modifications in technique imposed by the relatively smaller dimensions of bacteria would not seem beyond attainment.

Some of the methods most successfully used with the higher plants and which have not been thoroughly applied to the bacteria are those of differential staining, differential solubility, ash analysis, polarized light microscopy, x-ray diffraction, and hydration. Of these methods the first two have been most often attempted with bacteria and hydration studies not at all. Hydration methods involve the application of suitable reagents which cause specific swelling of the cell wall. A knowledge of the chemistry of the reagents used makes possible intelligent guesses as to the nature and organization of the responding material.

In a rather old study by Amann (1893) of various eubacteria illuminated

with polarized light, it was shown that the stained bacterial cell wall is *pleochroic*. This characteristic is also possessed by cell walls of higher plants. *Pleochroism* is the property possessed by crystalline and regularly oriented solids which produce different colors when the solids are rotated between crossed Nichols. Possession of this property indicates some crystallinity or regularity in the submicroscopic arrangement of the responding material.

The electron microscope has begun to expose the nature of the submicroscopic organization of the cell wall. In specimens of the genus *Bacillus* rod-shaped macromolecules embedded in a homogeneous ground substance have been photographed. In *Bacillus megaterium* the length of these macromolecules has been found to be 70 to 100 m $\mu$ . Such findings indicate that the cell wall of bacteria is not homogeneous, a fact which is corroborated by evidence from differential staining, electrophoresis studies, and serological analysis of the antigens in the cellular surface. The cell wall would seem to include proteins, carbohydrates, lipids, and in the case of gram positive organisms pentosenucleic acid. The ash content of the cell wall of the gram negative bacteria is negligible if published photographs of the results of the microincineration of *Spirillum volutans* are representative. Metals in the wall of gram positive organisms other than the magnesium associated with the pentosenucleic acid have not been studied. The cell wall is probably hydrated but the quantity of water present is not known.

The heterogeneity in chemical composition raises the possibility of a mosaic structure of the cell wall and cell surface. While students of the antigenic structure of bacteria are in the habit of speaking of the mosaic arrangement of the antigens of bacteria, there is little actual proof that the various antigens either are limited to restricted areas or occur in patches on the surface of the organism.

The chemical heterogeneity of the cell wall is suspected from results obtained by specific methods of staining which show that both positively and negatively charged groups are present. In the procedure recommended by Knaysi, tannic acid, an anionic mordant, is employed to render the cell wall stainable by a basic dye. On the other hand in the Dyar method a synthetic cationic surface active agent, cetyl pyridinium chloride, serves as a mordant for coloration of the cell wall by Congo red, an acid dye.

While evidence for the presence of hemi-cellulose or cellulose in the cell wall is ambiguous, there is no doubt of the presence of carbohydrate of some sort. Oxidation with periodate or periodic acid followed by Schiff's reagent as in the Pennington or Hotchkiss-McManus staining procedures results in coloration of the bacterial cell wall. This observation indicates the presence of polysaccharide with a characteristic —CHOH—CHOH— group in the 1,2 positions of constituent monosaccharide. The rigid framework of the cell wall may well be due to a polysaccharide of some kind with proteins



and lipids located between micelles of this material or in the interstices of the polysaccharide "skeleton".

The cellulose which has been described from species of *Acetobacter* should probably not be thought of as composing the cell wall but rather as constituting the specific substance of slime layer material. As is found for other cases of slime layer material, the production of cellulose by *Acetobacter xylinum* is in response to the presence of particular nutrients in the environment, hexoses, or substances convertible to hexoses being required. Other oxidizable carbon compounds such as pentose sugars, and various alcohols cannot serve in the synthesis of the cellulose.

Observation of changes in the surface properties of bacterial cells upon treatment with enzymes is a specific and increasingly favored method for studying the nature of the surface of bacteria. If the organisms have been freed previously of their slime layer the enzymatic changes must be attributed to action of the enzyme on substrates in the cell wall. Examples are the hemolytic streptococci in which trypsin has been shown to destroy a surface protein (so-called "M" substance) and *Staphylococcus aureus* in which the lipid content of the surface is reduced by the action of pancreatic lipase. It is interesting to learn from these experiments that an enzyme may destroy a component of the surface or cell wall without affecting the viability of the organism.

The presence of numerous kinds of chemical substances in the cell wall presents the problem of their possible role in metabolism. While it has not been uncommon to view the cell wall as a dead structure, this notion is being challenged. Botanists increasingly look upon the cell wall of plants as a living structure and as a part of the protoplast. The chemically heterogeneous nature and the relatively large fraction of the volume of the organism occupied by the structure fit in with this concept.

With yeast it has been possible to prove the presence of phosphatases in the cell surface. Thus when a yeast able to hydrolyze adenosine triphosphate (ATP) was exposed to ATP with radioactive phosphorus ( $P_{32}$ ) incorporated in the molecule, the ATP was found to break down with the accumulation of  $P_{32}$  in the culture filtrate but not inside the cells. That this distribution of the  $P_{32}$  was due to an enzymatic dissimilation of the ATP at, or on the cell surface, and not to the action of extracellular enzyme originating by secretion or autolysis, was indicated by the lack of enzymatic activity by culture filtrates. It has also been shown that low concentrations of uranium nitrate can inhibit anaerobic glycolysis by yeasts without any evidence of the penetration or accumulation of this poison within the cell. The conclusion drawn from this work is that the inhibition of fermentation involves enzymes in the cell surface. Also in the case of bacteria there are data which indicate the presence of enzymes at or near the cell surface. In Table 9

findings are recorded which indicate the presence of an adaptive sarcosine oxidase in the surface structure of *Pseudomonas aeruginosa*.

The mechanics of addition of new substances to the cell wall during growth and multiplication of bacteria is an entirely unexplored problem. Any proof of the living nature of the cell wall would suggest that this structure might be responsible for its own growth but this remains to be demonstrated. Apart from the problem of the role of the cell wall in assimila-

TABLE 9

*The effect of dodecyl sulfate on the oxidation of  $1.1 \times 10^{-2}$  M sarcosine by adapted and unadapted resting cells suspensions of Pseudomonas aeruginosa*

(The figures are mm<sup>3</sup> O<sub>2</sub> uptake; the autorespiration has been subtracted; 37°C, pH 7.8.)

MIN.	UNADAPTED			ADAPTED		
	Sarcosine	Sarcosine + drug	Inhibition	Sarcosine	Sarcosine + drug	Inhibition
			%			%
20	22	25	0	51	35	32
40	52	53	0	103	71	32
60	87	84	2	157	109	31
85	139	134	4	229	156	32
105	193	178	8	285	192	32
125	248	227	9	335	229	32
165	381	341	10	432	292	32

(From Bernheim, 1950.)

Note that the inhibition of sarcosine oxidation in unadapted bacilli does not exist from the moment of contact of the inhibitor with the organisms. Presumably the inhibited enzyme is located within the organisms, and the inhibition reaches a maximum only after sufficient time has elapsed for transport of the inhibitor to intracellular sites of action. On the other hand adapted organisms are inhibited immediately after the addition of the enzyme inhibitor, a result which is explained by assuming the adaptive enzyme to be present at the cell surface.

tory processes it is fundamental to ask how a rigid structure like the cell wall can be enlarged in the process of growth. While there are no data to discuss for the bacteria it may be helpful and suggestive for the student to briefly consider some of the findings from botany aimed at elucidating the nature of growth of the plant cell wall.

The energy for permanent elongation of the cell walls of plants is provided by the force of turgor pressure which increases during growth. The cell wall, though a rigid structure, is ductile so that the turgor pressure acts on a responsive plastic cell envelope. Actually the plant cell wall does not seem to possess a fixed ductility. Rather the *plasticity*, a term which is used



in the botanical literature in the same sense as ductility, varies during the growth phases of the individual cell. The plant cell wall includes submicroscopic aggregates or micelles of crystalline cellulose whose orientation is presumably responsible for the stretching or plastic properties of the structure. The plasticity is controlled by specific plant hormones called *auxins*. An auxin is defined as a hormone-like substance which causes permanent cell enlargement. The picture of cell wall enlargement may be summarized as follows. Accompanying growth there is an increase in turgor pressure resulting from the synthesis of new soluble materials and the uptake of water by the cell. Growth also is accompanied by the synthesis of additional and the release of normally bound and therefore physiologically inactive auxin. The released and mobilized auxin acts on the cell wall to increase plasticity. As a result the wall expands irreversibly in response to the increasing turgor pressure.

From these considerations it should be understood that the mere surface enlargement of the wall does not directly depend on the production of new cell wall material. Actually, if the wall is not to become indefinitely thin there must be eventually a synthesis of new material. The plant physiologists are not agreed as to where and how the new material is deposited. Some consider deposition to occur throughout the entire structure by intussusception of new material in the spaces between the micelles of cellulose. Others feel that the accretion of the new material might be limited to deposition of new layers along the inner surface of the cell wall.

With this background of information one might ask whether auxins have been found in bacteria or if auxins from plants can be shown to act on bacteria. Bacteria are known to synthesize plant hormones, and of these the auxin indole-3-acetic acid is produced in readily detectable quantities by a variety of eubacteria. Addition of indole-3-acetic acid in small amounts to *Escherichia coli* results in an increase in the population supported by a medium, while larger concentrations may cause a decrease. These observations prove indole-3-acetic acid to be a physiologically active compound for bacteria. Yet they do not indicate what role, if any, this substance plays in bacterial growth and cell wall elongation, since there are innumerable naturally occurring and synthetic compounds as well, which are not plant hormones but which show this same property of stimulation of total growth in low concentration and inhibition in higher concentrations. The bacterial synthesis of indole-3-acetic acid is a more significant finding. As a teleological argument one might claim that any normal constituent of organisms has some biological role, and from analogy, reason that the role of the compound in an unstudied situation is the same as for cases actually studied. But teleology and analogy are often fickle supports to depend upon in attempting to elucidate the truth. The progress of the botanists in defin-



ing the role of hormone-like substances in cell wall elongation has been made possible by the availability of a simple, reliable, and fairly precise method of study, the *Avena* coeleoptile test (see Went and Thimann, 1937). Unfortunately no similar technique has been suggested for study of the problem with bacteria. It is desirable in any future development of electron microscopy and x-ray diffraction as means for the study of the submicroscopic structure of the bacterial cell wall that an effort be made to note the effects of the addition of auxins.

### THE CYTOPLASMIC MEMBRANE

Lying next to the inner surface of the cell wall and separating the cell wall from the cytoplasm is the cytoplasmic membrane. This structure is a demonstrated discrete morphological feature of bacteria. It is a hyperchromatic structure and appears as a bright, sharp boundary with darkfield microscopy. By differential staining it is possible to color this structure differently than the cell wall and cytoplasm.

Whether the contact of the cytoplasmic membrane with the cell wall is a mere touching of adjacent surfaces or of a more intimate nature is indefinite in the case of the bacteria. With plant cells Hansteen-Cranner has presented some evidence to indicate that extensions of the plasma membrane may occur in the meshes of the cell wall. As a matter of fact this investigator would define the cell wall of plants as a colloidal network of cellulose in the meshes of which there are extensions of the cytoplasmic membrane. The occurrence of a colloidal system of lipids, proteins, and other non-cellulosic materials in the cell wall would thus be attributed to the intermingling of the cytoplasmic membrane with the cellulose "skeleton".

The earliest proof that a differentiated surface existed in bacteria similar to the cytoplasmic membrane of higher plants and animals was obtained from plasmolysis experiments. On retraction of the internal contents of organisms placed in a solution of high osmotic concentration, the cytoplasmic membrane pulls away from the cell wall and stands revealed as a refracting boundary surrounding the cytoplasm. From theoretical considerations it would be expected that the cytoplasm should have a differentiated surface. Cytoplasmic materials that reduce surface tension would tend to accumulate at the surfaces and thus result in an inhomogeneous distribution of chemical material between the surface and internal contents of cytoplasm. The result of this distribution has been succinctly stated by D'Arcy Thompson, "... the dissolved and adsorbable material has not only the property of lowering the surface tension, and hence of itself accumulating at the surface, but also has the property of increasing the viscosity and mechanical rigidity of the material in which it is dissolved or

suspended, and so of constituting a visible and tangible 'membrane' ". With this picture in mind and accepting the cytoplasmic membrane as a living structure, the common practice is to include this membrane among the alloplasmatic contents of the cell.

That the bacterial cytoplasmic membrane has properties distinct from those of cytoplasm can be shown by autolysis studies. At certain stages the lysis of the cytoplasm may proceed at a faster rate than that of the cytoplasmic membrane. As a result the optical density of the internal structure of autolyzing bacteria may be considerably reduced and the organisms appear empty and unstainable, while the inner surface of the cell wall remains in contact with a refractile structure which can still be stained by appropriate means. Exposure of tubercle organisms to sudden bombardment by a stream of high energy electrons has revealed that the cytoplasm may be disintegrated without a corresponding destruction of the cytoplasmic membrane.

The role of the cytoplasmic membrane in fission has been studied by a number of investigators who have not been in agreement in the details of their descriptions. How much the disagreement is a result of the use of different organisms and technics and how much to faulty technic and to misunderstandings of terminology is not clear. The findings do agree in assigning the cytoplasmic membrane an active role in the process of fission. An observation repeatedly recorded is that the visible evidences of fission are preceded by changes in the cytoplasmic membrane at the place where division will subsequently occur. By inward extension from a ring to an inner partition the cytoplasmic membrane grows and separates the cytoplasm. At this stage the developing transverse cytoplasmic membrane is often referred to as the cell plate. The cell plate eventually splits, the new transverse cell wall forming either as a single or double layer between the halves of the split cell plate. Before the separation of the two daughter cells is complete, formation of one or more new cell plates may be initiated and completed. As a result a rod-shaped organism may often be composed of a number of segmented portions, each one of which might be referred to as an individual, rather than considering the whole rod as a single organism.

### CYTOPLASM

The general appearance of the cytoplasm of bacteria depends upon the age of the culture and the conditions of growth. In the early stages of the growth of a culture the cytoplasm of the component organisms looks homogeneous both by light and dark-field microscopy. Granulation appears as the culture ages and becomes most prominent when the maximum rate of increase in the number of organisms has been passed. The granules which appear may be few or numerous and of varying size. In some species, as



the rhizobia and aerobic spore formers, the granules tend to fill the organism and may have only thin strands of basophilic cytoplasm visible between them.

Cytologists applying the techniques of micromanipulation and the specialized apparatus of biophysics to the study of plant and animal cells have made much progress in the understanding of the physical properties and organization of the cytoplasm of such cells. Unfortunately the small size of bacteria has prevented a concomitant accumulation of data on bacterial cytoplasm. Unlike the situation with plants and animals, statements on the nature of bacterial cytoplasm depend more heavily on reasoning from analogy and inference from data only indirectly related to the problem than on direct observation and experimentation.

Experience with ultracentrifugation of only one species, *Spirillum volutans*, has shown an order of viscosity for the cytoplasm similar to or only slightly higher than that usually observed for cells of the higher organisms. Curiously all the visible granules of this spiral-shaped organism collected at the centrifugal troughs indicating they had a higher specific gravity than the enveloping cytoplasm. This high relative density is unlike the experience with other organisms which show microscopically visible particulate structures such as mitochondria floating at the top of the centrifugally separated cytoplasm of intact cells.

Apparently there are at least two other ways in which bacterial cytoplasm may differ from that of other organisms. While it is not uncommon to observe Brownian movement of granules within what are probably cell sap vacuoles it is relatively rare to observe Brownian movement of granules directly embedded in the cytoplasm, a fact which may be related to the relatively greater density of the granules observed in *Spirillum volutans*. Also protoplasmic streaming has not been reported in the eubacteria. This unexpected lack, if true, is a challenging one since it may well indicate the absence of a mechanism providing energy in bacteria otherwise universally present among other kinds of organisms. The absence of this phenomenon among bacteria may be a clue to the function of protoplasmic streaming. Bacteria may be at a stage of evolutionary development where survival is independent of specific mechanisms for increasing the rates of diffusion within the body of the organism. Distribution of metabolites only by the kinetic motion of molecules (free diffusion) from the surface to the interior of bacteria may be rapid enough to meet the needs of these very small organisms. Larger organisms with greater distances to be traversed by diffusible substances might find free diffusion inadequate for mobilizing and distributing materials utilized at the necessary metabolic rates. Thus the auxiliary means for the transfer of metabolites which have appeared in the course of evolution have persisted because of their survival value in the



larger organisms. These means would include protoplasmic streaming as well as the vascular systems of multicellular organisms. Another interpretation of the absence of protoplasmic streaming in bacteria is possible. Streaming cytoplasm in bacteria would involve the movement of a small mass against the resistance offered by a relatively large enveloping surface area, and a great proportion of the energy of movement would be expended in overcoming the forces of friction. Unless one were to postulate the magnitude of the energy available to bacteria for protoplasmic streaming to be greater than that for other organisms, the rate of streaming in bacteria would be considerably reduced. For this reason it has been suggested that protoplasmic streaming may take place in bacteria but so slowly as to remain unnoticed. In view of these considerations it would be important for investigators who might happen to notice protoplasmic streaming in bacteria to report their observations.

Bacterial cytoplasm is a colloidal system in which water with dissolved soluble compounds acts as the external phase of the sol. There is some little evidence for the *thixotropic* character of the cytoplasm, that is, isothermally reversible sol-gel transformation. A fibrous type of protein (ellipsin) presumably responsible for the solation-gelation phenomenon in cytoplasm has been isolated from higher organisms. Similar studies have not been extended as yet to the bacteria.

One of the significant features of the organization of cytoplasm in general is that its components include metabolically active particulate bodies ranging from submicroscopic dimensions to the microscopically discernible mitochondria and the even larger plastids of plant cells. Differential centrifugation has been the chief means whereby these substances have been separated from the cytoplasm for *in vitro* studies. The association of enzymatic activity with cytoplasmic particulates is well known. Inasmuch as the particulates isolated cannot be considered to be in true solution in the disperse phase of the cytoplasm, their metabolic activity raises interesting questions as to the physical chemistry of biological processes involving multiphase or heterogeneous systems. Traditionally the biologist has thought of chemical and enzymatic activity as being limited to soluble substances and has tended to look upon the solid phases of protoplasm as being responsible only for mechanical properties or as providing the structural limits and boundaries for the localization of chemical function within the protoplast.

No large literature exists with regard to the presence of submicroscopic particulates in bacterial protoplasm. The presence of microscopic particulates possibly equivalent to mitochondria has been described in *Bacillus stearothermophilus* and *Mycobacterium* species. The dimensions of the bacterial organism itself are of the same order as some of the particulates of higher

organisms. It is of historical interest to recall that, prior to the application of biophysical tools to the problems of experimental cytology, an accepted hypothesis of the nature of mitochondria was that they were parasitic bacteria. This view is, of course, no longer seriously considered by workers in the field.

The most promising beginning on the problem of the physical nature of the components and organization of bacterial cytoplasm has been made with the electron microscope. There are already a few data available indicating the presence of macromolecular cytoplasmic constituents which may eventually prove to be equivalent to the particulates of the modern cytology of animals and plants.

The probable similarity of the organization of bacterial cytoplasm to the cytoplasm of other organisms is suggested by recent findings in histochemistry. It has been observed with all organisms including bacteria that phosphatases within the cytoplasm are localized in their distribution rather than uniformly dispersed. The compartmentalization of metabolic function within the cytoplasm of bacteria may also be suspected but not proven from the ability of bacteria to form within themselves visible granules of insoluble metabolic products. Such substances, referred to as inclusions, typically make their appearance in limited areas rather than throughout the entire cytoplasmic mass.

### INCLUSIONS

Non-living bodies deposited *de novo* in cytoplasm are called *inclusions*. The inclusions of bacteria are volutin, polysaccharides, lipids, and crystals of slightly water soluble inorganic materials such as calcium carbonate and sulfur. These inclusions are characteristic for a given species, but their actual presence and quantity vary with the conditions of growth. More intensive study of the variables affecting the formation of inclusions than has been done in the past should eventually lead to greater use of tests for the presence of inclusions in the identification of bacteria.

Volutin, also called *metachromatic* or *Babes-Ernst* granules, is a refractive basophilic substance widely distributed among bacteria, yeasts, and other thallophytes. Traditionally volutin has been identified by staining bacteria with toluidine blue or an aged solution of alkaline methylene blue and then searching for reddish bodies. Bacterial metachromatic granules are called volutin if they disappear upon exposure of the organism to dilute alkali (5%  $\text{NaHCO}_3$ ) or boiling water. The chemical nature of volutin is not completely understood, but it is generally stated to be partly, if not completely, pentosenucleic acid. It is not possible to say whether or not the volutin of different organisms is identical. Most recently metaphosphates which are metachromatic with toluidine blue have been isolated from



microorganisms. Cytological studies have not as yet been conducted to establish what relation, if any, these metaphosphates have to volutin.

The polysaccharide inclusions are detected by staining with iodine. Iodophilic material staining red-brown in color is usually labeled glycogen while blue staining material has been called *iogen*, *bacterial starch*, or *granulose*.

Lipid inclusions are identified by differential staining. Before Sudan black-B became available the usual practice was to apply the fat stain to organisms in wet preparations. With Sudan black-B a dried fixed smear may be employed. While the occurrence of fat granules is not limited to particular kinds of bacteria, they do appear to occur most regularly and abundantly in gram positive species. A globule taking a fat stain is not necessarily composed exclusively of lipid. Inasmuch as lipids would have the tendency to collect at exposed surfaces they might occur only in the periphery of granules. This suggestion is probably the explanation for the fat staining globules of *Spirillum volutans* which have a higher specific gravity than the enveloping cytoplasm. Such granules would appear to be composed chiefly of dense non-lipid materials coated by lipid.

The occurrence of cell sap vacuoles has also been noted in bacteria. That these structures arise *de novo* rather than from preformed and multiplying tonoplasts is not certain but is supported by recent observations made on the tuberculosis organism. While cell sap vacuoles of bacteria may be formed as consequences of normal metabolic activity it is important to realize that vacuolization of the cytoplasm of other organisms has been regularly observed to occur in response to toxic influences. As a group, fat solvents when introduced into an environment cause the appearance of vacuoles in exposed plant and animal cells. It would be desirable to learn if the same phenomenon occurs with bacteria. Inasmuch as the laboratory culture of bacteria often results in the harmful accumulation of lipid solvents and other toxic materials as products of fermentation (acetone, butyl alcohol, etc.) it would be instructive to know if these substances can act on bacterial cytoplasm as they do on other organisms.

Cellular movement of water also affects the vacuolization of cytoplasm. Thus Wiesner observed increased vacuolization under circumstances both of rapid gain and loss of water. Often biologists have tended to neglect the important observation of vacuolization accompanying water loss, and the subject has not been studied with bacteria.

The inclusions of bacteria are commonly considered to be reserve food substances. Evidence for this opinion rests on the observation of a relation between luxuriant nutrition and inclusion formation and the disappearance of inclusions during starvation. The intracellular accumulation of a product of metabolism would seem to be dependent both upon the solubility of the substance in water and its ability to rapidly penetrate the surface barriers



of the cell. Except for those substances involved in metabolic activity at the cell surface, impermeable, insoluble, or slowly diffusing slightly soluble materials would inevitably tend to accumulate within the cell rather than leave the cell. Consequently the mere accumulation of inclusions in response to an abundance of available food is not of itself proof of their reserve food nature but may be a result of rapid and excessive production of waste products. For an inclusion like calcium carbonate there is little reason to believe it serves a reserve food function. On the other hand with apparently metabolically oxidizable substances such as volutin, lipids, carbohydrates, and sulfur the problem is more complex. Parenthetically it may be remarked that if volutin granules are metabolically oxidized they cannot be composed exclusively of metaphosphate.

An alternative to the idea of the reserve food function of inclusions has been offered which derives from the theory of *metabolic shunts*. While most of the data in support of this theory have been drawn from work with fungi there is no doubt but that the concept has significance for the biology of bacteria. In a chain of reactions the overall rate of activity is dominated by the slowest reaction in the series. If excess food, usually in the form of a carbohydrate, is available the primary steps in its utilization may outpace the capacity of intermediate steps to bring the food to the final stages of catabolism as low molecular weight endproducts or for its assimilation into the intimate structure of the organism. As a result intermediate products of metabolism pile up in quantity and if they are only slightly soluble will precipitate out of solution. The excess accumulation of intermediate metabolites would also favor the transfer or shunting of material into alternative pathways of metabolism. Thus products normally found in only small quantities would form in large quantities or even predominate as the endproducts of metabolism. Depending upon their properties these materials would be excreted as in the case of water soluble alcohols and organic acids or would be precipitated within the cell as in the case of sulfur, volutin, lipids, and polysaccharides.

If we would arbitrarily define as a reserve food any intracellular substance which is preferentially used in periods of food scarcity, the theory of metabolic shunts permits a comprehensible explanation of the origin of these materials. However, acceptance of the origin of inclusions from mechanisms involving metabolic shunts should also mean the logical recognition of the fact that soluble *extracellular* products resulting from shunt metabolism are of the same nature as inclusions in their biological origins. Since most of these soluble products, like inclusions, can also be oxidized when other sources are unavailable they too would have to be considered reserve food material.

Paraphrasing Foster (1947) we might summarize three important bits of evidence relating shunt metabolism to the origin of inclusions:

1. Dilute media are rarely, if ever, populated by bacteria rich in inclusions.

2. If means are provided for eliminating the bottlenecks in intermediate metabolism then the products of shunt metabolism are reduced in quantity or entirely eliminated. This conclusion derives mostly from work with fungi rather than with bacteria.

3. The amount of organic acid formed per gram of sugar in the early stages of the growth of a culture is less than it is after the period of maximum rate of growth. Related to this finding is the common observation that "young" bacteria have a homogeneous cytoplasm in contrast to organisms in old cultures which are often intensely granulated (inclusions present).

In conclusion it is well to point out that the origin of the material of the slime layer of bacteria may be only a special case of shunt metabolism.

### THE NUCLEUS

The use of the term cytoplasm in the previous discussion of structure would imply a differentiation of the protoplasm of bacteria equivalent to that of other organisms. Actually the question of the existence in bacteria of a separable morphological entity equivalent to the nucleus has been much debated. Accumulating evidence does point to the existence of nuclei in bacteria, but unfortunately the evidence both old and new cannot be considered to have provided a definitive picture. The nucleus is both a morphological and physiological concept. A structure with given morphological characteristics may be defined as a nucleus, but the nucleus as a differentiated morphological structure has the leading role in the specialized mechanisms of heredity. It is this function, rather than structure, which defines the biology of any body we would label as a nucleus. The evidence of nuclear structure in bacteria is becoming increasingly convincing in its power to draw an analogy with the morphological nucleus of higher plants and animals although direct evidence of a role in hereditary processes for the described bacterial nuclei is absent. It is the thought and research devoted to this latter problem that will eventually yield substantial progress in bacterial cytology. Continued emphasis on the descriptive problems associated with the morphological nucleus will yield smaller and smaller rewards.

Unlike the case with other microbes and the cells of plants and animals, the direct observation of bacteria ordinarily does not show any differentiated structure which one might suspect to be a nucleus. The application of basic dyes tends to stain the whole bacterium. Thus the organism as a



whole possesses the basophilic character of normal nuclei. Nor does experience indicate that bacteria violate the laws of heredity. Based on such observations it was postulated early that the whole bacterial organism is the equivalent of a nucleus. In this view the hereditary units, or genes of modern cytology, were considered to be dispersed throughout the organism rather than collected within a limited structure. Not all students were content with this view, and so bacteriological literature has been rich in ideas as to the nature of the bacterial nucleus. Lewis (1941) has divided these notions into eight basic hypotheses, and may be consulted for full details.

Exposure of bacteria to x-rays and other high energy radiation results in harmful effects and death. It is possible to calculate what percentage of the quanta of energy bombarding bacteria results in a measurable biological effect. Such calculations have revealed that only a portion, as low as five per cent, of the hits are effective. Bacteria, therefore, seem to possess a limited area or zone sensitive to high energy bombardment. Drawing upon the experiences with the response of nucleated organisms to exposure to high energy radiation it can be concluded that the structure of bacteria includes material acting like nuclear substance and that this substance is not so finely dispersed as to render all portions of the organism equally sensitive to harmful radiation. These findings have lent renewed emphasis to the search for a morphological nucleus in bacteria.

The basophilic character of bacteria is due primarily to the presence of pentosenucleic acid, and when this material is removed the cytoplasm of the bacteria loses its intense affinity for basic dyes. The gradual recognition of these facts has been responsible for the great progress made in recent times in revealing the presence of nuclear-like bodies in bacteria. For the removal of the basophilic character of bacterial cytoplasm can be accomplished without an accompanying loss in stainability of nuclear structures by basic dyes.

The technics for unmasking nuclear bodies embedded in the basophilic cytoplasm have been of three kinds:

- 1) Acid hydrolysis. Exposure of bacteria to strong acid preferentially hydrolyzes the cytoplasmic nucleic acid, whereupon the application of various basic or so-called nuclear dyes and neutral stains will intensely stain bodies otherwise unrevealed in untreated organisms. The success of this technic apparently depends on the fact that the nucleic acid of the nucleus, primarily desoxypentosenucleic acid, is more resistant to acid hydrolysis than the cytoplasmic pentosenucleic acid. Since the relative resistance is only one of degree the choice of a time and temperature of exposure to the acid is important and must be empirically determined for the particular case. It also may be useful to stain at various acidic pH values since any



residual, partly hydrolyzed cytoplasmic nucleic acid will stain less intensely at acid values. Most commonly, hydrolysis has been done by heating in N HCl at 60°C for ten minutes. While this procedure reveals the presence of nuclear-like bodies, it is necessary to be critical of any conclusions drawn as to the appearance and size of these bodies in the living state. Obviously the procedure is harsh and may distort the true morphology of the organism. A particularly convincing study of the pitfalls in this general technic has been made by Heden and Wyckoff (1949) using electron microscopy to compare treated and untreated *Escherichia coli*. A better method of hydrolysis may be the use of cold perchloric acid as reported by Cassel.

2) Enzymatic hydrolysis. The basophilic character of the cytoplasm may be modified or removed by an appropriate enzyme. Crystalline pancreatic ribonuclease has been employed most commonly since the desoxypentose-nucleic acid of nuclear structures is not attacked by this specific enzyme. The use of an enzyme to reduce the basophilia requires that the organism be rendered permeable to relatively high molecular weight substances. In addition, pretreatment of the organism to disrupt the bonds between nucleic acid and proteins may also be necessary. This need is unfortunate since it precludes the reduction of the basophilia of *living* organisms by enzymes. As a result the morphology of the treated organisms cannot *a priori* be accepted as typical of the living organism.

3) Growth of organisms under conditions unfavorable to the development of a basophilic cytoplasm. The possibilities of this method were first reported by Knaysi and Baker (1947) who showed that germination of bacterial spores in an energy rich medium but lacking in nitrogen depleted the cytoplasm of the developing vegetative cell of basophilic substance and rendered nucleus-like bodies visible. Growth of organisms in carbohydrate rich and nitrogen and phosphorus poor medium prevents the normal synthesis of the basophilic substrate of cytoplasm. Yet as long as the organism will grow in such a medium its reproduction should be accompanied by an increase in nuclear material which will be stainable.

The use of this latter method also increases the possibility for observing nuclei in living bacteria by ordinary means and by phase contrast microscopy. In addition the difference in the nature of the cytoplasm is reflected in a reduction of its opacity to the electron beam of the electron microscope. As a result elegant photographs of differentiated and probably nuclear structures have been taken which would otherwise have remained unrevealed within bacteria grown on ordinary media.

Independent of the means of searching for bodies resembling a nucleus it is desirable to have criteria for establishing a particular structure in bacteria as equivalent to the morphological nucleus. The mere affinity of a

propelling mechanism. While such a train would tend to move forward as a whole the individual cars would assume a changing angular position at their flexible points of coupling.

A serious obstacle to accepting the concept of the origin of flagella as a result of motion is the increasing number of instances in which flagella have been shown to be present on bacteria grown in microcultures in which space for motility would be meager or absent. In addition it has been possible to study the chemical nature of the flagella separated from bacilli by the simple expedient of vigorous shaking followed by low speed centrifugation to remove the deflagellated organisms. Such studies have revealed that flagella consist of protein when the slime layer material of the parent species is essentially polysaccharide in nature. Even more interesting are the findings with x-ray diffraction which show the flagella to be composed of fibrous proteins. Such proteins have the fundamental structure associated with known contractile materials existing in muscle fibers.

While the origin of flagella from the slime layer is questionable, it is not possible to state whether the origin is in the cell wall or cytoplasm. Both notions have had their sponsors. The increasing evidence for considering the bacterial cell wall as an active metabolizing structure removes one objection to considering a cell wall origin for alloplasmatic structures such as the flagella probably are.

### THE BACTERIAL ENDOSPORE

Members of the family *Bacillaceae* and some species of *Vibrio*, *Spirillum*, and *Sarcina* are characterized by their ability to form endospores. As the name suggests an endospore is a resting or reproductive body formed within the bacterium. One organism gives rise to one endospore, and on transfer into a suitable environment the endospore germinates and yields a single vegetative cell.

Of all known living bodies bacterial endospores are the most resistant to toxic chemicals, desiccation, and heat. Their longevity has probably never been adequately tested, the science of bacteriology is too young! Suffice it to say that they have been found to be viable for as many years as individual bacteriologists within their own life spans have had the curiosity and patience to test for. It should be readily perceived that bacterial endospores present unique examples and problems for study by biologists and biochemists.

The endospore has had a major role in the historical development of bacteriology, for only after their discovery and recognition as exceptionally heat resistant bodies did it become possible to devise crucial experiments that decisively disposed of theories of spontaneous generation. Pure culture



studies in bacteriology had to wait upon an understanding of the properties of endospores. Practical problems of sterilization have had to be solved in terms of the capacity to rid an environment of these most persistent and hardy of living things.

While it is true that endospores are unusually capable of survival there are wide variations both among spores of different strains of a species, and different species, as well as among the individuals of a clone. In this regard the pattern of variation is not unlike that observed in other living forms though this fact is sometimes neglected. For example, it is a common procedure to heat a suspension of endospores to 80°C for five or ten minutes in order to obtain cultures free of living vegetative cells. Yet this temperature and time often will kill a large percentage of the endospores present even in the case of the most heat resistant types.

While the conditions necessary for the formation of endospores are not well understood and have been much debated, there are two observations which seem to have been made frequently enough with a sufficient variety of species to deserve the status of generalization. These are: 1) the optimum conditions for sporulation are like those for growth of the vegetative form, the permissible variation in the environment being within shorter limits than those for growth, and 2) the sporulation commences after the logarithmic period of most rapid vegetative growth. These facts are contrary to old teleological ideas and text book statements postulating harmful conditions in the environment as causes of sporulation. The thought was that since spores were resistant bodies they must have the biological function of carrying the organism through a period of hard times, and thus were formed in response to harmful conditions of environment. No critical research in the recorded literature supports this reasoning.

Actually the biological role of endospores is unknown. Since one bacterial organism forms only one spore which in turn yields one vegetative organism, the endospore cannot be said to be a device that multiplies the number of individuals of a species. In higher plants spores have a role in sexual processes or in phenomena of nuclear rearrangement. Thus there has been a stimulus to seeking a similar role for the bacterial endospore. No direct or indirect evidence exists for sexual processes in bacteria involving endospores. Cytological studies of dead stained material have sometimes been claimed to support the idea that endospore formation is an autogamic reproductive process. But present evidence does not permit the acceptance of *autogamy*, the fusion of nuclei of the same cell, as the underlying phenomenon associated with sporulation. The descriptions of autogamic processes by various authors do not agree in detail. Nor has there been definitive proof that the objects called nuclei are not inclusions, and cytologists differ in their opinion of which staining bodies within spores may properly be



called nuclei. Genetic studies lend no support since ultraviolet and x-ray irradiation affect both endospores and parent vegetative forms in the same way as far as the rate of phenotypic expression or mutation are concerned. The mutation data have been interpreted to mean that both parent vegetative cells and endospores are uninucleate and probably haploid. In a process of autogamy one would expect to observe some organisms in a diploid phase.

The most recent suggestion has been to assign to the bacterial spore a role as an agency for the aerial distribution of the species. Evidence for this view is poor and entirely speculative. As one objection, spore-forming species do not grow directly exposed to the atmosphere. Hence their opportunities for being swept up by wind currents are completely fortuitous rather than predetermined by their circumstances of growth. They grow in bodies of water, in soil when the moisture content and relative humidity are not favorable to pulverization, in the intestinal tract, and in marine sediments. As a matter of fact these latter organisms exist at depths of the sea removed, for eons of geologic time, from any contact with the atmosphere of the earth. To rescue this hypothesis we would have to invent another, namely, that marine spore-forming bacteria are descended from terrestrial types. However, this procedure is scientifically poor since it depends for support upon the invention of another and unverifiable hypothesis. In this particular case the hypothesis of a terrestrial ancestry for marine species in the absence of any evidence is totally unacceptable. As a rule evolution has proceeded from marine forms toward terrestrial forms. There is no reason to consider marine spore-forming bacteria as an exception. It has also been claimed that endospore adaptation to aerial distribution is shown by their reduction in size and weight, but this is a misquotation of fact. Bacterial spores uniformly show a greater specific gravity or density than homologous vegetative bacilli. The smaller size of bacterial endospores would give them only a small advantage over vegetative cells for purposes of flight through the air. With Cook (1932) we would agree that some bacteria "form spores because they form spores." The need for new ideas remains.

The cytology of the formation of endospores has been studied most effectively by means of motion picture photomicrography. The portion of the cell in which the endospore forms first increases in both opaqueness to light and electrons and in viscosity. The differentiated area is referred to as the *forespore* and may undergo rapid and reversible changes in volume before the final highly refracting rigid endospore with its characteristic coat makes its appearance. After the endospore is formed the remainder of the mother cell gradually disintegrates.

The structure of the endospore includes a spore coat which is composed

of one or two layers, an outer thin coat of low refractive index, and an inner denser wall apparently responsible for the high index of refraction of the spore. The internal contents appear to be homogeneous. In germinating endospores, bodies resembling nuclei may be differentiated, and these are located at the periphery of the cytoplasm right beneath the endospore wall.

Upon transfer to a favorable environment the endospore will germinate and yield a single vegetative cell. The appearance of the spore during germination varies with the organism and is characteristic for each species. The position and way in which the spore coat is broken is easy to observe and is significant for purposes of species identification. An early sign of germination is swelling of the spore accompanied by a diminution of refractive index, and the spore, normally impermeable to dyes, becomes penetrable. In the spores of gram positive organisms the internal cytoplasm as a whole or only granules or chromatinic bodies may stain gram positive depending on the species. The same is true for the growing vegetative cell after it ruptures the spore envelope.

Germination of endospores is favored by conditions conducive to vegetative growth except that a lower osmotic pressure is preferred. As heterotrophic organisms, the spore-forming bacteria require oxidizable organic compounds for germination to proceed. In addition, in certain species unknown heat labile dializable substances have been shown to favor germination. Such factors are not extracted from boiled spores and must presumably occur in nature apart from spores. In non-synthetic media inhibitory factors for germination, including high molecular weight fatty acids, have been reported. Of particular interest is the common observation that the pre-heating of endospores at sublethal temperatures but above temperatures at which vegetative growth will take place favors the subsequent germination. No explanation for this phenomenon is available.

Endospores have nothing unusual about their chemical composition. The water and ash contents are like those of vegetative cells, except for a higher percentage of calcium; the nitrogen content is somewhat higher than in vegetative cells, and they possess a full complement of the naturally occurring amino acids. There are no obvious clues in the composition to suggest a basis for the unique physiological properties of endospores.

Studies of antigenic structure prove that endospores like all other living things are composed of a mosaic of antigens. While some of the antigens are characteristic of the spore alone, others are found in common with the parent vegetative form. There is no evidence that any particular antigen characterizes all endospores; on the contrary, the endospores of different species are antigenically distinguishable. There also is evidence that differences between the antigens of spores and homologous vegetative cells include polysaccharides as well as proteins. These findings mean that the



formation of the endospore must be accompanied by synthesis of new organic material. Since sporulation of vegetative bacilli can be induced in distilled water the sources for synthesis of specific spore material must be endogenous. The appearance of the forespore then cannot be a mere matter of a separation of a portion of chemically unmodified vegetative cell material. These findings also suggest that in seeking for chemical differences to explain the biological nature of the endospore it will be more productive to think in terms of differences in the properties of individual organic constituents rather than in gross differences in the overall content of particular

TABLE 10

*Stability of spores of Bacillus metiens as measured by studying the resistance to disinfectants of spores stored for varying lengths of time*

DRIED SPORES EXPOSED TO N/NaOH AT 30°C			SPORES IN BUFFERED WATER EXPOSED TO HYPOCHLORITE†		
Storage time	Viable spores/ 0.01 grams	LT99*	Storage Time	Viable Spores/ ml	LT99
<i>days</i>	$\times 10^6$		<i>days</i>	$\times 10^6$	
1	91	43	1	20	96
51	90	42.5	6	15	90
114	65	44	10	18	99
129	97	38	30	25	100
150	99	42	240	13	102
Mean.....	88.4	41.9		18.2	97.4
Average Devia- tion.....	$\pm 9.2$	$\pm 1.6$		$\pm 3.4$	$\pm 3.5$

\* Lethal time in minutes to kill 99% of exposed spores.

† 1,000 parts per million available chlorine at pH 11.1; 20°C.

(From Levine, 1952.)

kinds of substances, such as water, lipids, proteins, etc. In any case studies of antigenic structure have provided the only unquestionable proof of qualitative differences between the chemical compositions of endospores and vegetative cells.

The endospore as a resting body does not manifest any great metabolic activity although this does not mean that endospores do not possess an active metabolism. On the contrary a variety of enzymes including catalase and gelatinase and a number of respiratory processes have been described. Low as it is the endogenous respiration of moist spores is readily evident and measurable with the Barcroft-Warburg respirometer.

The few studies done on the respiration of endospores suggest that they contain a full complement of the same kinds of enzymes found in vegetative cells. However, the quantities may be much lower, thus six per cent of the



cytochrome, and fifty per cent of the haematin content of vegetative cells has been reported to occur in the endospores of *Bacillus subtilis*.

In testing disinfectants or in comparing factors over an extended period of time it is often desirable to have a stable stock suspension of organisms. Because of the viability of bacterial spores over extended periods of storage they may be ideally suited for the purpose. In Table 10 data in support of this use of bacterial spores are presented.

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## CHAPTER VII

# Surface Properties of Bacteria

Life has been viewed as the continuous adjustment of the internal environment to the external environment (T. H. Huxley). As the genius of Claude Bernard aptly put it, "*La fixité du milieu intérieur est la condition de la vie libre.*" A condition for maintaining the internal environment seems to be its partial physical separation from the external world. A barrier must be interposed across which all communication between the internal and external environments takes place. In the bacterium the cell wall and cytoplasmic membrane acting together or alone may be regarded as forming this barrier.

As do all three-dimensional objects, bacteria must possess an external surface by which is meant an outermost area in immediate contact with the external universe. This surface is not a passive element resting without impression in an environment but has characteristics that dynamically influence the structure of the medium which bathes it. It is the purpose of the following discussion to elaborate on the properties of the bacterial surface and the role of that surface in the maintenance of the "*milieu intérieur.*"

The surface structure of bacteria has a variety of properties such as the ability to be wet by various liquids, electrical charge, "stickiness" or a tendency to cause neighboring cells to cohere and to act as surface for the adsorption of molecules. In the last case bacteria can change the distribution of solutes in water by presenting additional surface at which molecules tending to reduce free energy in a boundary layer will preferentially accumulate. All these are properties normally expected for aqueous colloids. As a result it is possible to understand these surface properties of bacteria in terms of common and well developed physical-chemical concepts and to use the specialized tools of the physical chemist for their study. Consideration of the physical-chemical nature of the bacterial surface inevitably has led to attempts to elucidate the mechanism for the maintenance of the osmotic relations of the organism, for the regulation of the exchange of material between cell and environment, and for the defense of the cell against specific natural methods of destruction such as phagocytosis.

The nature of the problems to be considered permits the study of the physical-chemical properties of the bacterial surface without the necessity for identifying the actual morphological structures involved. Thus the



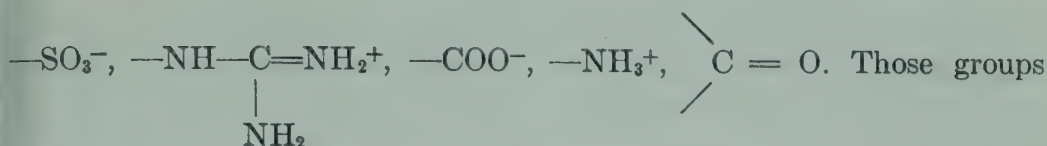
characteristics of the surface responsible for such properties as the electrophoretic mobility and the selective permeability of the cell can be delineated in physical-chemical terms without any knowledge of actual morphological structure. The identification of morphological structures with the structure responsible for the surface properties can be treated as a separate problem to which the physical-chemical data contribute knowledge. That is, aspects of the chemical and physical organization of morphological structures involved in the surface properties of the organism can be deduced from the description of the surface properties. This generalization holds since morphological structure must account for the physical-chemical picture. Of course, the reverse is also true, but the inadequate state of the present day knowledge of morphology does not permit much interpretation of the physico-chemical nature of bacterial surfaces based on morphological data.

### THE WETTING OF BACTERIA

It is well known that bacteria are wet by water although this observation is so common that its importance unfortunately may be neglected by the student. Wettability is important since this property brings the organism in close contact with water and substances in solution and is fundamental to an understanding of many biological phenomena. A solid surface wet by water has had the water spread over it so that the surface is intimately bathed by the liquid, and any capillary spaces exposed at the surface are filled with water. A surface wettable by water is spoken of as being *hydrophilic* or water loving, while one over which water will not spread is said to be *hydrophobic* or water hating. Surfaces may vary widely in the extent of their hydrophilic or hydrophobic properties.

The chemical nature of the surface determines its wetting properties. The cell surface is composed of a heterogeneous mixture of chemical groups some of which attract water and others of which tend to repel water. Such groups are also spoken of respectively as hydrophilic and hydrophobic or *polar* and *non-polar*.

In general, the hydrophilic groups are polar, meaning that they possess ionizable or charged components. Such charged structural units tend to dissolve in water or associate with it, as in the process of hydration. Apparently the charged structural elements have a strong affinity for water and other liquids of high dielectric constant because water is also a polar substance and possesses a small negative charge on the oxygen atom with compensating positive charges on the hydrogen atoms. The existence of both charge types in the water molecule permits the electrical attraction between water and charged groups of either positive or negative sign. Among the hydrophilic or polar groups may be included,  $-\text{OH}$ ,  $-\text{NH}_2$ .



with charges indicated as ionic are, in general, capable of producing the greatest effect on water since they possess charges of relatively large magnitude.

On the other hand, hydrophobic or non-polar structures, as the second name implies, possess no significant charges. As a result, there is little attraction for polar structures and so water does not readily associate with them. The more important common non-polar groups are the hydrocarbon radicals, e.g.,  $-\text{CH}_3$  and  $-\text{C}_6\text{H}_5$ . Actually, of course, the list of known chemical groups forms a spectrum ranging from the highly polar ionic groups to the non-polar hydrocarbons, and the contribution to surface wettability varies accordingly. Surfaces relatively high in polar groups are wet by water, and those high in non-polar groups are not.

A characteristic interfacial tension exists at any boundary between phases. Those cases of greatest interest in bacteriology are the liquid-air and liquid-solid interfaces. In the former situation the terms surface tension or surface energy are commonly employed to indicate the differences in the properties of the liquid surface when compared with the body of the liquid as a whole.

In the body of a phase, a molecule is subjected to fields of force exerted by its neighboring molecules. While these forces fluctuate, they fluctuate so rapidly that the resultant over a finite time is zero. Hence, a molecule may move through the body of a liquid without doing work against the forces exerted by its neighbors because the field is uniform throughout its path. However, when a molecule is brought to the liquid surface, it must move against unbalanced forces because it is no longer symmetrically surrounded by the molecules of the homogeneous liquid phase. It follows, then, that there is a resultant force perpendicular to the surface that acts on the surface molecules. Thus the molecules at an interface possess extra energy by virtue of their position because of the work done in bringing them to the surface. This statement is, of course, an expression of the third law of thermodynamics. Such energy of position is given the name surface energy and is responsible for the peculiarities of interfaces.

One of the important phenomena in surface chemistry is the spreading of liquids on the surfaces of solids or other liquids. In general, the process occurs spontaneously only when it results in a reduction of the free energy of the system. Beyond this simple statement there is no completely unified and satisfactory theory of spreading.

In the spreading of a liquid on a liquid the mobility of the lower phase



(liquid) is essential since it seems to be molecular movement in the lower liquid that disperses the upper liquid, if spreading occurs at all. A powdered, surface active solid like stearic acid floating on a liquid might be spread by the same mechanism. Apparently a drop of oil spreads on water by association of a molecule of the oil with a molecule of water and diffusion of this complex. An increase in the available surface brought about by this method allows more oil molecules to contact the water and spreading continues. The spreading complex of oil and water tends, by viscosity or cohesion, to carry along adjacent oil molecules and thus cause spreading of the drop as a whole.

When a solid and liquid are in contact, the liquid may or may not spread over (wet) the solid. The same condition of free energy reduction must be met as before, but measurements of the free energy of solid surfaces cannot be made. However, the contact angle between the liquid and the solid indicates the extent of spreading. When the angle of contact becomes zero the spreading is completed. In other words, the liquid wets and flows over the solid. The exact mechanism of this phenomenon is obscure since the immobility of the surface of the solid seems to prevent any process resembling that occurring on liquids. Present hypotheses are all incapable of accounting for the various observations and a new approach seems necessary.

The wetting properties of the bacterial surface can be studied experimentally. As a result it is possible to distinguish differences in the wetting properties of various organisms and to study the effects of variables including changes in the conditions of growth. The first such studies made by Mudd and associates utilized a method of observation with the dark field microscope. In this procedure a suspension of organisms in water or some aqueous medium is placed between a coverslip and slide. While the organisms are being observed under the microscope a drop of liquid immiscible in water is introduced at the edge of the coverslip. To the extent that it is immiscible with water the solvent is drawn by capillarity under the coverslip with a separate phase boundary and sweeps the water ahead of it.

On the chance approach of a bacterium to this moving boundary one of several things can happen. The organism may remain in the water phase and be swept across the field in the water ahead of the moving water-test solvent boundary, the organism may move into the interface and stay there, or it may enter the test solvent phase. Similarly a suspension of bacteria may be vigorously shaken in a two phase liquid system consisting of water and a test solvent. After shaking the phases are separated, aided by low speed centrifugation, if necessary, and the distribution of the organisms in the phases and at the interface is noted.

The behavior of bacteria observed by either of these methods is dependent



on the balance of hydrophilic and hydrophobic groups in the bacterial surface and on the interfacial tension existing at the liquid-liquid boundary. The interfacial tension is related to the mutual solubility of the phases, which is a function of the nature and quantity of polar and non-polar groups in the molecule of the test solvent.

Whether the bacteria remain in the water phase or move into the interface or the test solvent might be predicted from a knowledge of the interfacial tensions at the liquid phase-bacterial surface boundaries. Unfortunately, there is no direct method for measuring the interfacial tension at a solid-liquid interface. However, the angle of contact between a liquid and solid is directly measurable, and by the application of the simple geometry of the resolution of forces to angles of contact it is possible to explain how the bacteria can move out of the water phase in which they are wetted into an interface or into another phase in which respectively they will be wetted only in part or not at all by the water phase. The fundamental relation between angles of contact and wetting has been neatly phrased by Maxwell: "When a solid is in contact with two fluids the surface of the solid cannot alter its form, but the angle at which the surface of contact of the two fluids meets the surface of the solid depends on the values of the three surface tensions. If the tension of the surface between the solid and one of the fluids exceeds the sum of the other two tensions, the point of contact will not be in equilibrium, but will be dragged towards the side on which the tension is greatest". This concept and its consequences are illustrated in Figure 21.

The experimental findings have shown that bacteria tend to be caught in high tension water-organic solvent interfaces. Thus the wetting properties of a given organism will vary with the nature of the solvents being tested and the quantities of solutes affecting the mutual solubilities of the immiscible phases. The student should note that solutes tending to increase the mutual solubility will have the effect of decreasing the interfacial tension.

Of even greater interest is the finding that bacteria vary among themselves in their ability to be wet by non-polar solvents. This indicates significant differences in the distribution of polar and non-polar chemical groups at the surfaces of bacteria. Of all organisms the acid-fast species have the most hydrophobic surfaces as indicated by their tendency to move from an aqueous suspension into non-polar solvents such as cyclohexane and olive oil. As a generalization, it might be said that gram negative species act as though they have the most hydrophilic surfaces, while gram positive organisms have less and acid-fast species the least hydrophilic character.

The entrapment of organisms at a liquid-air interface is obviously favored by a surface constitution predominantly hydrophobic in nature. No doubt this explains in part the normal tendency for many organisms including

acid-fast species to grow as a membrane or pellicle at the surface of broth media. In this regard the fact that an organism is heavier than water and should sink is compensated for by the sum of the buoyancy of the organism

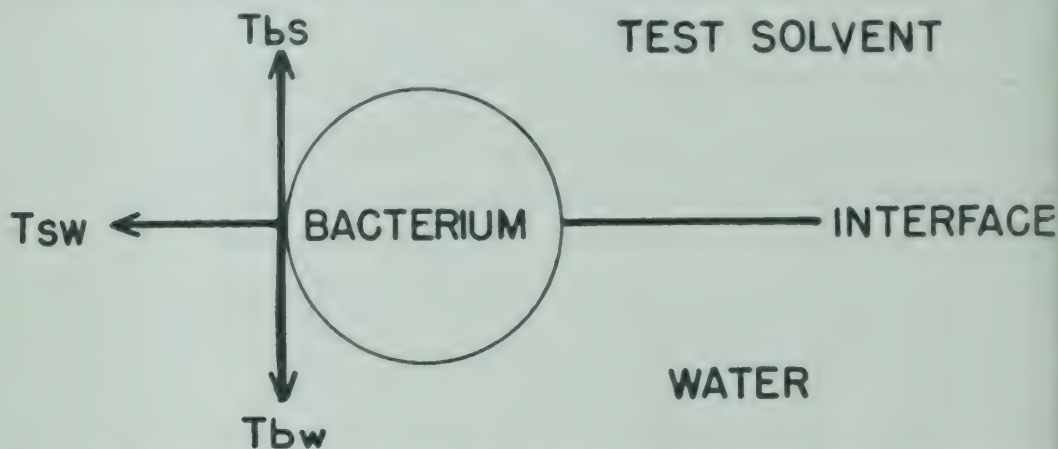


FIG. 21. Illustration of the wetting of bacteria depending upon the relative strength of the various surface energies involved.

$\angle w$ —angle of contact of water and bacterial surface (Measured from the water-test solvent interface to the tangent of the water-bacterium interface)

$\angle s$ —angle of contact of test solvent and bacterial surface (Measured from the water-test solvent interface to the tangent at the test solvent-bacterium interface)

$T_{bw}$ —interfacial tension at the bacterium-water interface

$T_{bs}$ —interfacial tension at the bacterium-test solvent interface

$T_{sw}$ —interfacial tension at the test solvent water interface

By the resolution of forces it can be predicted that if:

$T_{bs} > T_{bw} + T_{sw}$  the water phase will be pulled around the bacterium. The organism will be completely wetted by water.  $T_{bw} > T_{bs} + T_{sw}$  the test solvent phase will be pulled around the bacterium. The organism will be completely wetted by the test solvent.

$T_{bs} < T_{bw} + T_{sw}$  or  $T_{bw} < T_{bs} + T_{sw}$  the organism will be trapped in the water-test solvent interface. The organism will be pulled to a greater or lesser extent into the water phase than the test solvent space depending on whether the bacterium-water interfacial tension is less or greater than the water-test solvent interfacial tension.

(Adapted from Mudd and Mudd, 1924)

A little thought will make it clear that the events illustrated are in accordance with the third law of thermodynamics. In other words, the behavior of the organism will be such as to result in a decrease of free energy at the surface of the bacterium to the minimum possible value.

due to its displacement of liquid and the vertical component of surface tension (fig. 22). This expectation is experimentally verified by a reduced tendency of bacteria to grow as pellicles when surface tension reducing agents are added to liquid media. A most significant advantage of this

finding has been taken by Dubos and associates who add non-ionic surface active agents such as the synthetic detergents Tween 80 and Triton A20 in order to effect a dispersed growth of the acid-fast tubercle bacillus in broth media.

Variations in the chemical constitution of an organism under different conditions of growth and of mutant strains of a species may be reflected in differences in surface composition. As long as such differences shift the balance of hydrophilic and hydrophobic groups at the cell surface they may be detected by wetting experiments. Thus a simple and effective means is available for study of important problems of fluctuation in the chemical

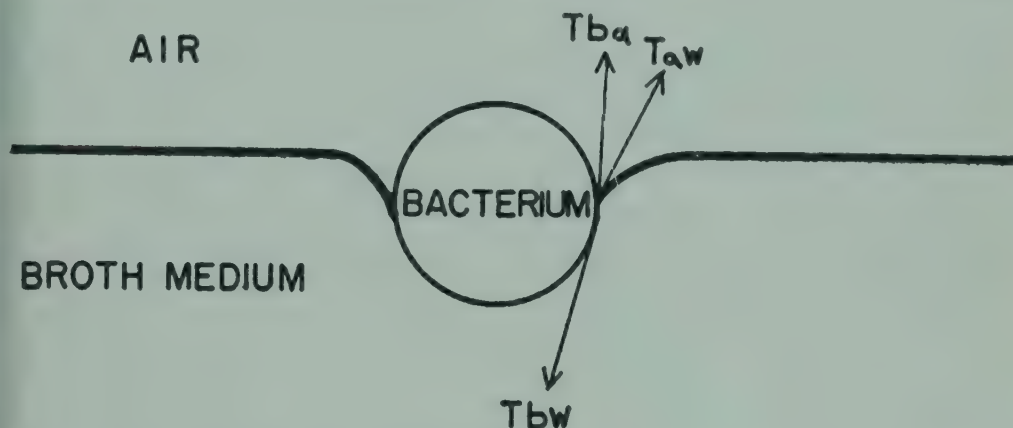


FIG. 22. Bacterium lying at a liquid-air interface.

The organism will float when  $T_{bw} > T_{ba} + T_{aw}$ . It will be displaced below the horizontal plane of the air-water interface where an equilibrium will exist between the vertical tension acting on the organism and the buoyancy due to displacement of water by the floating organism.

composition of cells. In this regard the more hydrophobic strains of tubercle bacilli have been found to be the more virulent strains. Such a finding suggested that the more virulent strains were producing greater quantities of a hydrophobic substance somehow related to virulence. This clue has been productive of significant studies on the problem of bacterial virulence. It has also permitted an explanation of why the more virulent tubercle organisms characteristically grow in long cord-like arrangements while the less virulent or avirulent strains more often show the individual organisms in colonies to be in an irregular arrangement.

The phagocytic phenomenon has also been explained on the basis of the same type of analysis developed in connection with interfacial tensions and the wetting or spreading of liquids on a solid surface. The displacement of the aqueous medium-solid particle interface by the solid particle-phagocyte interface is treated in terms of the balance of forces existing between the



tensions at these boundaries. Engulfment of solid particles such as bacteria by a phagocytic white cell is thought to occur when the tension of the aqueous medium-solid particle is greater than the sum of the tensions at the solid particle-phagocyte and water-phagocyte interfaces. Variables affecting these tensions would concomitantly influence phagocytosis. Phagocytosis then may be thought of as the spread of a fluid solid surface (the phagocyte) over a solid particle (the bacterium) in response to forces of interfacial tension.

### THE ELECTRIC CHARGE AT THE SURFACE OF BACTERIA

As early as 1904 bacteria were observed to bear an electrical charge. The nature of the charge is demonstrated by observing the tendency of bacteria to migrate in an electrical field. Under the usual conditions of growth and at pH values near neutrality bacteria will accumulate at the anode and migrate from one electrode to the other on reversal of the polarity indicating that their surfaces carry a negative charge. The nature of this charge is interpreted in the same way as that of colloidal particles in general. The movement of charged particles in an electric field is called *electrophoresis*. Often the terms *cataphoresis* and *anaphoresis* are employed in order to specifically designate whether the direction of movement is toward the positively charged (anode) or negatively charged electrode (cathode). The difference in potential between the medium and the bacterial surface has been called the *zeta potential*. The quantitative measurement of surface charge or zeta potential must be strictly controlled if the results of various investigations are to be comparable. For this reason the standardized method of Moyer (1936) has been used by most bacteriologists since its publication.

As indicated, the electrophoretic migration of particles in an electric field depends upon the presence of charges on those particles. The necessary charges may arise in several different ways which may be operating singly but which more often are combined in some way. Obviously, when present in a structure, the fixed charges of ionized groups will contribute quite significantly to the total. In the usual case the ionizations are influenced by the pH of the medium with charges appearing or disappearing as the pH is altered. Hence the direction and velocity of migration in a field will depend upon the sign and number of the ionized groups which is controlled in turn by the hydrogen ion concentration of the medium.

If droplets of mineral oil in a dilute solution of sodium chloride are subjected to an electric field, they are observed to migrate toward the anode and behave as though negatively charged. However, mineral oil certainly possesses no structures capable of ionization under such conditions, so the

charge must originate by some mechanism other than ionization. When a chloride ion due to its kinetic motion approaches the oil drop, it induces a separation of charges in the surface atoms of the oil, and this separation gives rise to an electric moment. This induced moment in turn attracts and tends to bind the chloride ion to the droplet. Frequently the energy of such binding is much greater than the thermal or kinetic energy of the ion. Thus the ion is firmly adsorbed to the particle and by its presence confers a charge on the particle with which it then migrates.

Since ions of both signs will be available for adsorption, the process must be at least partially preferential. It may be stated in this connection that the magnitude of the binding force varies inversely with the dielectric constant. Water happens to have one of the highest dielectric constants and greatly reduces the adsorption of ions from its solutions when compared with other solvents. In a similar connection the strength of the forces involved in adsorption varies inversely with the distance between the adsorbent and the adsorbed particles. Hence the less water between the particles involved, the stronger will be the adsorption. It follows, then, that hydrophobic surfaces not readily wet by water will show a maximum polarization (charge separation) and charge attraction. Furthermore, the less an ion is hydrated, the greater in general will be its adsorption by such a mechanism. Ordinarily, negative ions are less hydrated than cations, and, in the case considered previously, the result is a relatively greater adsorption of chloride than sodium ion, yielding a net negative charge to the adsorbent. Probably the polarization effects described contribute somewhat to the charge of bacteria since, as will subsequently be brought out, a part of the cell surface consists of lipid material and should be hydrophobic in character.

Ion pair formation will also be a factor in the magnitude of the net charge on a particle. Ions tend to associate by electrostatic attraction between charges of opposite sign. In the case of sodium chloride and similar univalent salts, ion pair formation is quite low because the energy of electrostatic attraction is not sufficiently great to prevent separation of the ions by thermal action. However, the energy of binding of an ion to a particle depends upon the total number of charges of sign opposite to that of the given ion. This condition suggests that proteins or cells with many charges of a given sign will have a pronounced tendency to show ion pair formation with the ions of salts, buffers, and such. Since charges of opposite sign are adsorbed by this means, then the net charge on the particle will appear to be lower than expected from knowledge of the actual number of ionized groups making up the surface of the particle. The nature of the salts present is of importance with divalent ions being more strongly adsorbed than monovalent ions. In practice, ion pair formation can cause profound



changes. Hence, care must be exercised in comparing electrophoretic data taken under different conditions of solution.

Occasionally, specific chemical reactions may occur by which ionic charges are added to or masked on a particle. These situations are probably less common than the other charging processes, but the results are likely to be profound. Very often isoelectric particles altered by such a mechanism may have the isoelectric point so shifted as to be unmeasurable. Ordinarily, the original material cannot be recovered after such a process, and visible modifications may occur indicating that chemical changes have taken place.

Hydrogen bonding can result in the attachment of charged structures with a quite significant alteration in the surface charge. In many cases, one cannot distinguish this process experimentally from ion pair formation although theoretically hydrogen bonding ought to contribute considerably. With chloride and most of the other ions derived from single atoms, hydrogen bonding obviously can be excluded as a factor.

It will be noticed that the foregoing discussion has involved only the "surfaces" of particles, inferring that the interior of a particle is unimportant in electrophoretic migration. A simple experiment has shown that this supposition is almost completely correct. If a mixture of different kinds of particles are suspended in a dilute neutral salt solution and subjected to an electric field, the particles will migrate at characteristic velocities, partly toward the cathode and partly toward the anode. However, when one adds neutral gelatin this material coats the particles, and they all migrate at the same rate and all toward the anode. Therefore, one is forced to conclude that the surface charge is the major controlling factor and that any charges buried within the particle do not greatly, if at all, influence electrophoresis. From this classical experiment it may be likewise concluded that the size and shape of the particle (within rather wide limits) do not seriously affect the direction and velocity of migration.

In addition to the net surface charge, factors which do control the velocity of migration in a field include the temperature, the strength of the field or potential gradient, viscosity of the medium, and the proportion of the electric current carried by the particles to the total current. These factors must be rigidly controlled or account must be taken of them by calculation of the migrational velocity using an appropriate equation.

No attempt will be made here to discuss the detailed theories or methods of electrophoresis since both are beyond the scope of this text. The principles outlined above will provide a basis for understanding the purpose and nature of the electrophoretic studies carried out on bacteria. For a more extended understanding of the scope and techniques, the reader is referred



to "Electrophoresis of Proteins and the Chemistry of Cell Surfaces" by Abramson, Moyer, and Gorin (1942).

Apart from some spirochetes bacteria normally show a negatively charged surface. There is no good evidence of polarity of the individual organism since bacteria will usually change their direction of movement in an electrical field in response to a reversal of electrodes without flipping over or first making an obvious turning motion. A body with a fixed polarity would have to make some such motion in order to maintain the necessary orienta-

TABLE 11

*The variation in the electrophoretic mobility of individual organisms of different strains of staphylococci*

STRAIN	NO. ORGANISMS TIMED	MEAN TIME*	STANDARD DEVIATION	COEFFICIENT OF VARIATION	ELECTRO- PHORETIC VELOCITY*
		(seconds/100 $\mu$ )		(%)	( $\mu$ /sec/volt/cm.)
AuB.....	28	9.45	0.90	9.5	2.0
HA.....	40	8.09	1.04	12.9	2.5
Fs.....	32	9.12	1.22	13.4	1.8
M4.....	40	6.78	0.59	8.8	2.6
AuS.....	28	6.46	0.57	8.8	2.9
JH7.....	48	5.85	0.45	7.8	4.0
CT.....	28	4.22	0.41	9.7	4.2
F19.....	40	4.21	0.16	3.9	5.6

\* Cells suspended in M/75 phosphate buffer pH 7.4. Cultivated on veal infusion agar for 18-22 hrs. at 37°C.

(From Verwey and Frobisher, 1940.)

tion of its oppositely charged ends always facing toward the respective attracting electrodes.

Under fixed conditions of growth and measurement the electrophoretic mobility of a given strain of organisms is found to be a reproducible value. Actually the individuals in a clone will not all have exactly the same mobility, so it is really a mean value quoted as characteristic for the strain. The extent of variation of individuals within a clone is not the same for all strains. Data for the staphylococci are typical and are listed in Table 11. They show an order of variation no greater than commonly is encountered for other characteristics of biological populations. It has also been observed that while the mobility value for a given strain is consistent, the value within the species may vary widely. The variation between different strains is continuous rather than discontinuous. Thus in a study of 84 strains of staphylococci under similar conditions, a range of 2.2 to 6.6  $\mu$ /sec./volt/cm.

was recorded with all possible intermediate values occurring. In this study a variation of  $\pm 0.2 \mu \text{ sec./volt/cm.}$  was considered significant. The same continuous nature of the variation has been noted for the different dissociants and mutants obtainable from a given strain. However, the rough phase dissociants usually have a greater electrophoretic mobility than the smooth dissociants.

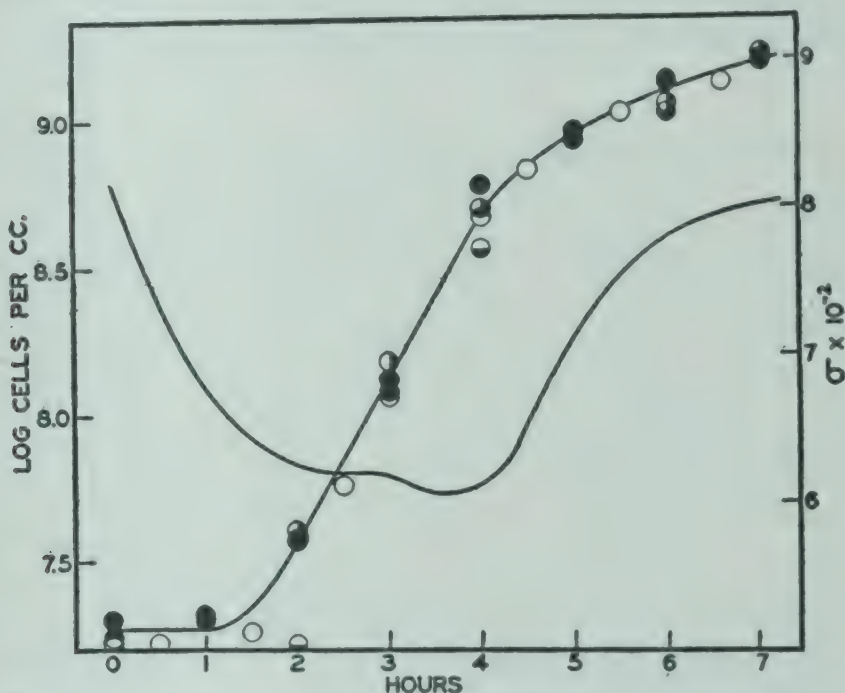


FIG. 23. The mean charge density and phase of growth of *Escherichia coli*. The circles are plate counts and establish the growth curve. The second curve shows changes in the charge density during multiplication in the various stages. Charge density is plotted using the right hand ordinate where  $\sigma$  is units of charge per unit of surface area.

(From Abramson, Moyer and Gorin, 1942)

The surface charges of bacteria vary with the age of a culture, as in the case of *Escherichia coli* (fig. 23). Upon inoculation of this organism into fresh peptone broth, growth is accompanied by a drop in the electrophoretic mobility to minimum values coinciding in time with the early phases of growth of the culture. The mobility values begin to rise again after the period of the greatest rate of multiplication is past and reach a maximum and fairly constant value after the population of viable cells in the culture has become stabilized in the so-called maximum stationary phase of the growth cycle of the culture.

As expected from theory the electrical charge of bacteria varies with the

ionic strength<sup>1</sup> and pH of the suspending medium. At the isoelectric point the potential will drop to zero, and this pH value is often referred to as an *isopotential point*. On the acid side of an isoelectric protein or other biological surface the charge will be positive, while it will be negative on the alkaline side. Bacteria, therefore, generally carry a negative charge at neutral pH because their surface components include amphoteric materials with isoelectric points at acidic pH values. For most bacteria the charge becomes positive when the pH of the menstruum is dropped below the range of 2.0–3.5. A few organisms such as some species of *Salmonella* have been found to have a negligible electrophoretic mobility over a range of pH values (5.5–7.0). However, most organisms show a continuously increasing negative charge toward a maximum alkaline value somewhere between pH 9 and 11. A reversal of charge has been noted not only at low pH values but also under intensely alkaline conditions. Both *Bacillus cereus*, a gram positive species, and *Escherichia coli*, a gram negative species, have been reported to undergo an unexplained reversal to a positive charge above pH 13.5.

There have been studies aimed at the correlation of the electrophoretic mobility of bacteria with their biological properties, but on the whole such attempts have been disappointing. At one time much attention was devoted to defining a relationship between the electrophoretic mobility and the virulence of pathogenic species. Now there is no doubt that the surface charge of bacteria will vary if the surface chemistry varies in such a way as to affect the kinds and quantities of ionizable material at the surface. A virulent encapsulated smooth strain of pneumococcus may very well have a different electrophoretic mobility than an unencapsulated rough avirulent pneumococcus. But here the correlation is not between the virulence of the organism and its surface charge. Rather, the correlation is between virulence and the presence of some particular chemical component at the cell surface. There is no evidence that the presence of capsular polysaccharide material on a pneumococcus increases virulence because of any change it may induce in the potential difference at the cell surface. In this case then we are dealing with a causal association and not a correlation of virulence and electrophoretic mobility since the increased virulence and accompanying change in mobility result from the same cause.

<sup>1</sup> The term ionic strength is applied to a function of concentration that is weighted for the charge on the ions. In situations in which the charge of the soluble ions as well as their number is important the ionic strength is always used. It is defined by  $\mu = \frac{1}{2} \sum c_i z_i^2$  where  $\mu$  is ionic strength,  $c_i$  is the molar concentration of any individual ion, and  $z_i$  is the charge on that ion. The product  $c_i z_i^2$  is summed for all ionic species present in the solution.



This example illustrates the difficulty in attempting to assess the role of surface charge in a given biological phenomenon. Unless supporting proof can be offered, the mere isolated observation of a systematic change in electrophoretic mobility and a biological property is no reason to accept a cause and effect relationship. The value of such an observation may really lie in providing a clue as to the chemical nature of a substance at the cell surface which is actually related in a causal way to both the biological phenomenon and the charge on the bacterial surface. It is this latter consideration which makes electrophoretic studies a powerful tool for bacteriology in spite of any present failure to relate electrophoretic behavior as a causal phenomenon in biological processes.

Electrophoretic studies are expected to contribute to our knowledge of the presence of polar and amphoteric substances at the bacterial surface. Once a knowledge of the normal surface charge is established, variables may be manipulated and their influence studied. From a knowledge of the nature of the variable, and its effect on the electrophoretic mobility, particular aspects of chemical structure and physical organization may be deduced. An example of such a methodology has been the study of the effects of benzenesulfonyl chloride on the electrophoresis of bacteria at various pH values in order to establish the presence of amino and imidazole groups at the cell surface. Various enzymes have also been used. Thus changes in the surface charge accompanying the application of trypsin have been interpreted as indicating the presence of digestible protein at the surface (fig. 24).

Explanations of the nature of bacteriostasis and killing effects may also be suggested by electrophoresis studies. The knowledge that cells may be killed by heat without any change in electrophoretic mobility tells us there has been no effect by the heating on the charge of the proteins exposed at the surface. While this observation does not reveal how the heating procedure killed the organisms, it does exclude certain possibilities from further consideration.

Suggestive evidence of the nature of disinfection can sometimes be obtained by techniques which are still in the developmental stage. The variation of the electrophoretic mobility when *Escherichia coli* is exposed to sulfanilamides reveals that the drug is reacting with aromatic groups. The findings also lend support to the hypothesis that the sulfanilamides act as bacteriostatic agents because they compete with an essential metabolite, *p*-aminobenzoic acid, for available cellular receptor sites. In this case it is the similarity in the effects of the sulfanilamides and *p*-aminobenzoic acid on electrophoretic mobility which is suggestive.

Though non-polar groups by their nature do not contribute ionizing structures to the surface charge of bacteria, electrophoresis remains a

powerful means for studying the relative proportion of surface area occupied by such groups. Since the bacterial surface is small the possible number of charges has an upper limit related to the saturation of the surface with ionizing polar groups. The application of specific chemical reagents to a surface to tie up particular ionizing groups can reveal the nature and amounts of these groups by measurements of a change in surface charge.

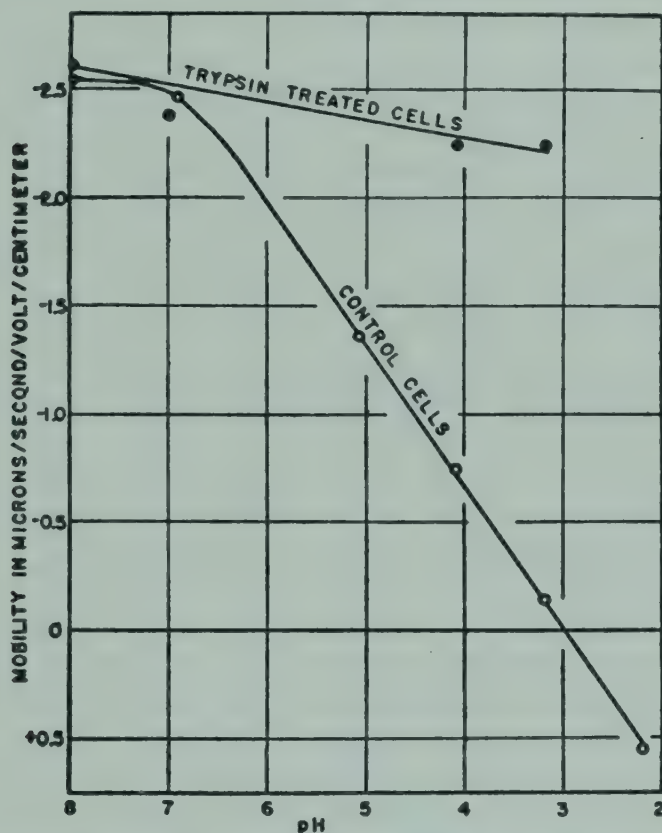


FIG. 24. The electrophoretic mobility-pH curves of normal *Micrococcus aureus* cells and cells treated with trypsin.

(From Dyar, 1948)

If the summation of the charges due to the detectable groups adds up to the actual surface charge, the area occupied by these groups subtracted from the total surface area is a measure of the area occupied by non-polar groups. Actually, at the present time serious practical difficulties enter into this kind of analysis.

A promising method being developed for obtaining clues of the chemical nature of biological surfaces is the study of the effects of synthetic ionic surface active agents on electrophoretic mobility. Synthetic ionic surface active agents or detergents are compounds composed of a non-polar portion



or "tail" combined with an ionizable polar portion or "head" and in aqueous solution tend to aggregate into high molecular weight micelles. Cationic detergents possess a positive charge while the anionic detergents have a negative charge.

Bacteria exposed to these materials will have their electrophoretic mobility modified. Cationic detergents in proportion to their concentration will cause a decrease in the negative charge of bacteria, and with sufficient detergent the charge may actually reverse and become stabilized at some positive value. The presumed explanation of these events rests on the solubility of the non-polar tails of the detergent in surface lipids. The ionized polar head "sticks out" of the lipid surface because it is not wet by the lipid, and contributes to the surface charge of the bacterium. Thus a cationic detergent will reduce the negative charge and, if present in sufficient quantities on the cell surface, can reverse the surface charge. Obviously the maximum shift in the electrophoretic mobility in the presence of some limiting concentration of detergent is a relative measure of the lipid area of the exposed surface of the organism. This conclusion is valid since the uptake of detergent by the surface will be a function of detergent concentration and of the amount of lipid in the surface available for combination with the detergent. Studies of this kind have revealed the same general picture for different organisms in spite of differences in their initial charges and in the concentrations of individual detergents at which there is a reversal of charge.

Study of isolated lipids in emulsions exposed to detergents can reveal the maximum electrophoretic mobility attainable with a detergent in a given buffered solution. This value would also be that expected of the bacterial surface if the surface were composed entirely of lipid saturated with the detergent. The difference between the actual mobility of the normal organisms and the maximum mobility in the presence of detergent is an index of the proportion of the surface occupied by the lipid. Thus in the case of *Staphylococcus aureus* with a normal mobility of  $2.3 \mu/\text{sec}/\text{v}/\text{cm}$ , the maximum change found with the anionic surface active agent sodium tetradecylsulfonate solution was  $0.4 \mu/\text{sec}/\text{v}/\text{cm}$ . Since extracted lipid from the same organisms migrated at a maximal value of  $6.4 \mu/\text{sec}/\text{v}/\text{cm}$ , when exposed to the same detergent the percentage of lipid at the cell surface is

$$\frac{0.4}{6.4 - 2.3} \times 100 \sim 9.8\%$$

Obviously in such an experiment it is necessary to evaluate the effect of the size of the suspended lipid particles since the surface area of these particles will control the quantity of adsorbed detergent and therefore the total charge per particle. Under such circumstances large droplets will migrate faster than small ones.

That the presence of lipid at the bacterial surface is involved in the effect



of the detergent on electrophoretic mobility has been shown by employing the lytic enzyme lipase. The electrophoretic mobility of normal cells remains unchanged after exposure to the lipase. But the change in mobility affected by the detergent is reduced following treatment of the cells with lipase. This finding depends upon the hydrolysis of the lipids by lipase and the subsequent loss of these materials from the cell surface. After such treatment the organism shows a diminished affinity for detergents.

### THE STABILITY OF BACTERIAL SUSPENSIONS

Bacteria suspended in broth media and other liquids often are observed to clump. The resulting aggregates will sediment at rates predicted by Stokes' law. In the case of organisms growing in filaments and chains, chance entanglement of strands will be responsible for flocculation, but this phenomenon can hardly account for agglutination of single cells nor the agglutination effected by diverse chemical agents. The conditions causing and otherwise influencing the agglutination of bacteria have been the subject of numerous studies. While many problems remain incompletely resolved, it has been productive to think of the agglutination in terms of colloid chemistry.

The balance of hydrophilic and hydrophobic groups at the exposed bacterial surface is presumably responsible for the apparent behaviors of the organisms, sometimes as lyophilic<sup>2</sup> and at other times as lyophobic colloids. In a given aqueous menstruum the stability of a suspension of colloid particles, and probably of bacteria too, is related to:

1) Hydration (tendency of each organism to surround itself with the liquid phase thus preventing surface contact of the organisms):

- a. of ionizing polar groups
- b. of non-ionizing polar groups
- c. of salts or ions adsorbed at the cell surface

2) Electric charge (on the chance close approach of similarly charged organisms there would be mutual repulsion).

The greater the zeta potential and solvation by a liquid of high dielectric constant such as water, the greater the tendency for particles to remain apart. Conversely any variable introduced into an environment which causes a reduction of hydration and/or of the surface charge will act as a destabilizing agent.

The common dehydrating reagents such as alcohol, acetone, and concentrated solutions of salts such as ammonium sulfate used to "salt out" pro-

<sup>2</sup> *Lyophilic* and *lyophobic* correspond to hydrophilic and hydrophobic except that the solvent is generalized. In a lyophilic colloidal system the internal and external phases are intimately associated and there is no well-defined demarcation between the phases.

teins from solution are all capable of flocculating bacteria. Actually, in spite of this general similarity to the hydrophilic proteins, bacteria often act more as hydrophobes. Like the lyophobic metallic sols bacteria frequently are:

- 1) Agglutinated by freezing,
- 2) Agglutinated by relatively low concentrations of univalent, or traces of polyvalent ions of opposite charge,
- 3) After agglutination resuspended only with difficulty.

This last similarity in behavior to a lyophobic sol may not be real in all cases since it might be due to an irreversible chemical change. The behavior is analogous to a lyophobic sol only if the original cause of agglutination has not resulted in a permanent change in the chemical nature of the cell surface by a process of denaturation, extraction of a constituent, or other possible means.

The role of the electric charge of the bacterial surface may be inferred from the fact that organisms will clump at pH values where the electrophoretic mobility is reduced to zero (at the so-called isoelectric point). Changes in the nature of salts present and of the ionic strength of the suspending medium affect simultaneously and similarly the isoelectric points as measured by electrophoresis and the acid agglutination of bacteria. The presence of "protective colloid" such as serum protein or gelatin will, like the simpler electrolytes, also influence these isoelectric points. The bacteria tend to acquire the isoelectric characteristics of the added colloid. Table 12 presents some data illustrating the effects of gelatin in various buffers on the acid agglutination of *Escherichia coli*.

If similar electric charges do tend to hold bacteria apart, then mixtures of organisms of opposite charge should flocculate by mutual neutralization of the charges. This expectation has been realized experimentally for two strains of the *Bacterium leprosepticum* which normally agglutinate at different pH values and the finding that a mixture agglutinates in the intermediate pH zone. Furthermore, basic proteins such as protamines and papain, the latter with an isoelectric point at pH 9, will agglutinate bacteria in solutions of low ionic strength.

Actually, it is not necessary to reduce the surface charge to zero in order for agglutination to occur. Agglutination can occur over a range of low values of potential usually of the order of  $\pm 15$  millivolts. In other words, it has been observed that the experimental manipulation of a variable does not result in agglutination until the zeta potential is reduced to values within this critical range. This zone of potential difference between the bacteria and suspending medium in which agglutination will occur has been called the *critical potential*. The width of the zone of critical potential is a function of the nature and concentrations of electrolytes present. It is of



great interest that bacteria agglutinate in a *zone* of critical potential in spite of their individual possession of a low but definite, similar electric charge. In this connection it is also interesting to note that organisms in the early stages of the development of a culture are least susceptible to acid agglutination and yet also show the lowest electrophoretic mobility at this same time. Among the *Enterobacteriaceae* a large number of organisms in their smooth dissociative phase have been noted to possess a zero zeta potential over a wide range of pH values, and yet they form stable homogeneous suspensions.

These observations are proof that forces other than electric charge are involved in agglutination phenomena. In these cases the bacteria are acting

TABLE 12

*Effect of buffer salts and gelatin on the flocculation of Escherichia coli at varying pH values*

CONCENTRATION OF GELATIN	pH VALUES AT WHICH FLOCCULATION OCCURS		
	No buffer	0.0125M acetate buffer (Range studied: pH 3.2-5.6)	0.0125M lactate buffer (Range studied: pH 2.4-4.7)
0	1.6-3.0	3.2	None
1:4,000,000	1.6-3.0	3.2	None
1:400,000	1.6-3.4	3.2-3.5	None
1:40,000	1.3-3.4	3.0-4.1	3.0
1:4,000	4.0-4.6	3.8-5.0	None
1:400	4.6-5.0	4.7	None

Isoelectric point of gelatin is at pH 4.7.

(Data taken from Eggerty and Bellows, 1922.)

as typical hydrophilic colloids. In general, the precipitation of hydrophilic sols is not possible by merely reducing the charge on the particles to zero. Desolvation must also occur if the sol is to flocculate. Likewise dehydration in the sense of the loss of loosely associated water at the surface is one suggested explanation of how similarly charged bacteria and proteins in solution may be precipitated. An interesting case is the precipitation of tobacco mosaic virus by bovine serum albumin at pH values at which both materials are on the acid or alkaline sides of their isoelectric ranges. The virus is visualized by the original investigators as having less of an affinity for water than does the serum albumin, and the serum albumin competes successfully for the solvent with the result that the virus is dehydrated and separates from solution. Slight amounts of neutral salts enhance the precipitation of the virus in the presence of the albumin.

In summary, the observations with bacteria indicate a complex situation.



Depending on their age, dissociative phase, circumstances of growth, and the nature of the suspending medium, they may act as either lyophilic or lyophobic colloids.

Some rough phase dissociants of organisms are notoriously difficult to suspend homogeneously in nutrient media or dilute salt solutions. Such organisms when growing in broth will spontaneously agglutinate independently of any acid production. This latter factor of acid production is important because lowering the pH of the medium to the isoelectric point can result in the agglutination of cultures which otherwise give homogeneous suspensions.

With the *Salmonella* it has been shown that spontaneous agglutination is due to the presence of cellular constituents soluble in lipid solvents. Thus the alcoholic extraction of such organisms renders them stable in suspension in dilute salt solution. The alcohol extracted substances when re-added to the homogeneous suspensions of extracted organisms cause reagglutination. From smooth culture phase organisms apparently similar materials can be isolated. However, the addition of these extractable substances from either R or S phase organisms (both causing "salt sensitiveness" of the R phase cultures) does not render S phase cultures spontaneously agglutinable. Possibly this is evidence for the presence of stabilizing (hydrophilic?) groups in the smooth culture, absent in rough culture. This explanation is only tentative since the original experiments were not designed to exclude the possible effect of salts on the zeta potential.

Acid-fast bacteria, like other organisms, have a critical potential of agglutination. In spite of the relatively great difficulty experienced in wetting these organisms with water, lipid extraction to the extent of loss of acid-fastness has been reported not to result in a change in their isoelectric point as measured by electrophoresis nor to influence the effect of salts on their agglutination. These observations suggest that the extracted lipids probably constitute a minor portion of the surface of these organisms.

In the foregoing discussion agglutination has been considered only as a problem of reducing the forces that normally keep organisms apart. But if agglutinated bacteria are studied critically it becomes apparent that the organisms do not fall out of suspension and lie next to one another in merely chance positions. Further, Brownian motion produces erratic motion of clumps as units and appears to be an insufficient force to cause single organisms to break loose from the clumps. If agglutinated organisms are shaken, the clumps are not observed to break up readily into separated, individual organisms. Actually, one may have to apply a great deal of energy in order to separate the individuals within a clump. These observations indicate that there is a force, or forces, holding the agglutinated organisms together. Agglutination then, must involve not only the over-

coming of forces of repulsion which tend to keep cells apart but also involve forces of cohesion tending to pull organisms together. Whether homogeneous dispersion or agglutination occurs under a particular set of circumstances must depend upon the balance of the cohesive and repulsive forces.

A simple, ingenious method for measuring the cohesive force acting on bacteria has been devised by Northrop and De Kruif. Two glass plates are covered with a film of bacteria and placed together with the coated sides touching each other. The force required to pull the plates apart is a measure of the cohesive force. The bacilli coated plates are immersed in test solutions to determine the effect of the solutions on the cohesive force. By such a procedure variables like pH and salt concentration have been found to affect the cohesive force as well as the surface charge of bacteria. If both the cohesive force and zeta potential are reduced, agglutination will not occur. However, if the cohesive force is not changed, agglutination will occur upon continued reduction of the zeta potential of bacteria. Electrolytes in concentrations of the order of 0.1 M or less affect the surface charge of bacteria proportionately more than the cohesive force, while in high concentration the cohesive force may be much reduced relative to the surface charge.

Since the method of measurement gives no clue to the nature of the cohesive force, and since Northrop and De Kruif did not hypothesize as to the nature of the cohesive force, some investigators have ignored and others challenged the existence of a cohesive force between bacterial cells. But this lack of understanding does not invalidate the concept. Many natural phenomena are recognized which can be measured quantitatively with accuracy in spite of a lack of basic knowledge of the nature of the phenomena. The case of gravitational force is an example *par excellence* of this intellectual dilemma. It can be measured accurately, it can be used to successfully predict the motions of bodies in space, and yet the nature and origin of the gravitational force remains a mystery.

It is possible that the cohesive force involves surface energy. Apart from forces of repulsion, the distribution of surface energies will determine the state of aggregation of bacteria in suspension. When the forces of repulsion decrease to quantities no longer overcoming the net pull of the interfacial tensions, the organisms will clump. In this situation the cohesive force is the difference between the interfacial tensions existing at the bacteria-solvent interface and the contacting surfaces of the bacteria. If the interfacial tension at the bacteria-water interfaces is greater than the interfacial tension at the contacting surfaces of the bacteria, the organisms will tend to be pulled together and will cohere. This follows since the natural tendency is for a system to assume the state resulting in a minimum of free surface energy. Unfortunately, it is not possible to say whether these concepts of



surface energy account in whole or only in part for the cohesive force as measured by Northrop and De Kruif. The reason for this is the present inability to experimentally measure all the surface energies existing in a bacterial suspension, particularly the surface energy at the area of contact of bacterial cells in clumps.

*Agglutination by antibody.* The theory of agglutination by specific antibody has been the subject of much speculation stimulated in part by a large body of conflicting experimental data. Suffice it to say that, after Bordet demonstrated the role of small amounts of neutral electrolytes in inducing the agglutination of bacteria previously exposed to specific antibody, a colloid chemical concept was applied to serological agglutination. The antigen-antibody reaction is visualized as a two-stage reaction with the first stage one of specific reaction of antigen with antibody. The specific antibody accumulating at the surface of the bacteria results in a system which tends to act like a hydrophobic sol so that agglutination follows as a second reaction in the presence of small quantities of electrolytes. Many recent students of antigen-antibody reactions have preferred an alternative explanation, a lattice hypothesis or one-step reaction theory. The antibody molecule is viewed as being "bivalent" or "multivalent" and builds up aggregates by directly acting as a bridge linking antigen molecules or bacteria in suspension.

Quite recently the role of non-specific lipids such as cholesterol in aiding serological agglutination and precipitation has been rediscovered and emphasized. It is difficult to see how the delipidation of antisera, a process which does not reduce the combining power of antibody but does reduce the flocculation phenomenon, can be explained by the lattice hypothesis. Observations of this character emphasize the necessity for maintaining an open mind on the relative value of the older colloidal-chemical theory for explaining the aggregation accompanying antigen-antibody reactions in spite of the present tendency of many serologists to accept the one-stage lattice hypothesis *in toto*.

### OSMOSIS

All living organisms possess the capacity to regulate the interchange of materials between their internal structure and the external environment. This regulation is achieved by the interposition of a selectively permeable barrier across which all exchange must take place. The barrier is permeable to water and is selective in its behavior toward the passage of other substances. Such a structure in the cells of plants and animals has been identified with the cytoplasmic or plasma membrane. In bacteria the cytoplasmic membrane has likewise been thought of as the selectively permeable barrier.

The presence of such a differentially permeable barrier between two



solutions of different total concentration leads to the phenomenon known as *osmosis*. This process may best be defined as the diffusion of a solvent (usually water) through a membrane. If water at a given pressure be separated from an aqueous solution at the same pressure by a membrane impermeable to the solute, water molecules diffuse through the membrane in both directions. However, the rate of diffusion is greater from the direction of the pure water because the respective rates will be directly related to the two concentrations of water. As a consequence there will be a net flow of water into the solution and we say that osmosis is taking place. It should be emphasized that the pressures were assumed to be the same on both sides of the membrane and that under this condition water passes the barrier in the direction of the solution. Furthermore, the pressure differential on the membrane is either extremely small or zero.

When the solution is under a pressure greater than that on the water, then the relative rate of diffusion of water molecules from the solution is increased in proportion to the additional pressure applied. If the pressure is made sufficiently high, water will diffuse equally in both directions and the system is in a dynamic equilibrium called *osmotic equilibrium*. Plainly, the concentrations are not at equilibrium and the pressures are quite different with a considerable pressure differential being exerted on the membrane.

With these facts in mind we may now define the osmotic pressure of a solution as that pressure which must be applied to the solution to bring it into diffusion equilibrium with respect to the solvent. This concept is concerned only with a property at a particular equilibrium condition and is somewhat analogous to the freezing point of a material which also denotes the condition of an equilibrium state without any relationship to the actual temperature of the material. In other words, when a system is not at osmotic equilibrium, then the pressure on the solution is not the osmotic pressure.

Considering such a system now in a slightly different light, one realizes that the rate of diffusion of solvent through the membrane in a given direction depends upon the number of collisions of solvent molecules with the membrane which depends in turn directly upon both the pressures on the system and the concentration of solute. At osmotic equilibrium the excess pressure on the solution must compensate exactly for the low concentration of solvent and one will be a measure of the other. Extending this notion a little further, one concludes that the higher the concentration of solute the higher is the osmotic pressure of the solution. This general relationship is expressed quantitatively for *dilute* solutions by means of the equation:

$$P = \frac{RTw_2}{VM_2}$$

where  $P$  is osmotic pressure,  $R$  the gas constant,  $T$  absolute temperature,  $w_2$  grams of solute dissolved in the volume  $V$  of solvent, and  $M_2$  is the molecular weight of the solute. In very dilute solution  $V$  may be taken as equal to the volume of solution.

An important concept now becomes evident; namely, that the osmotic pressure depends upon the number of moles of solute particles dissolved and not upon the nature of those particles. An ion acts as a particle. Thus the effective osmotic concentration of an ionizable substance at any given moment is the sum of all the individual ions and molecules present. Thus a solution of a mixture of solutes with a given total molar concentration has the same osmotic pressure as a solution of a pure solute of equal molar concentration.

As might be anticipated, the above equation provides a means for the determination of the molecular weight (or particle size) of solutes when membranes impermeable to them are available. The method is extremely useful for substances in the molecular weight range of several thousand upward including particularly the proteins and many polysaccharides. Since osmotic pressure measurements are able to count only the number of particles present and cannot reveal the presence of more than one apparent molecular species, the method requires supplementation for the establishment of homogeneity in order that one may be confident that the determination of the molecular weight is for the single desired species.

Unfortunately, bacterial cells are so large relative to ordinary molecules that the osmotic pressures of their suspensions are too small to measure, and other methods must be used to determine the "molecular weights" of these particles. However, the concepts of osmosis and osmotic pressure are important in considering the individual bacterium, since these organisms possess differentially permeable membranes. These membranes completely surround the interior of the bacterium causing osmosis, with the direction and extent of movement depending upon the relative osmotic concentrations of the interior solution and the suspending medium.

When osmosis transfers water into the cell the increase in the volume of the inner solution necessarily is restricted by the rigidity offered by the enveloping membranes and a pressure develops in the cell. Under normal conditions of growth the outer structures of the cell are able to withstand this pressure which, while it may be high, represents but a small total force because the area affected is so extremely small.<sup>3</sup> Apparently the membranes

<sup>3</sup> To avoid confusion on this point, it is well to recall that pressure is force per unit area. While the osmotic pressure applied by the cell wall to the internal phase of the bacterium may be great at equilibrium, the total force actually exerted is really quite small because of the small area concerned. The resistance of the cell wall to distortion is the source of the force concerned and therefore, ultimately of the pressure which



of the cell are thus able to withstand the rather high osmotic pressure that arises from the presence of the large non-diffusible molecules dissolved in the cellular liquids. If cells are suspended in media of abnormal concentration, then collapse or rupture of the cells may occur depending upon the relative osmotic concentrations and the mechanical strength of the membranes.

Since cells may appear distended or turgid from the pressure developed inside them, the term *turgor pressure* is sometimes applied to the pressure in the cells. In general this term will be equivalent to the osmotic pressure only when equilibrium is reached and water is diffusing at the same rate into and out of the cells. It should be kept in mind, however, that any change in the volume inside the cell will alter the osmotic concentration. Hence the attainment of actual equilibrium will depend upon the osmotic concentration of the final solution and not upon the osmotic concentration of the cellular liquid before stretching occurs.

Size considerations preclude direct pressure measurements on bacteria, and several factors make estimation of turgor pressures difficult and of doubtful value. In the first place, the permeability properties of the bacteria are not known with any completeness nor are the osmotic concentrations within the interior of these organisms readily determinable. The latter becomes particularly troublesome because of the problem of solubility and aggregation of solutes within the cytoplasm.

Evidence indicates that many of the large molecules making up the protoplasm may be combined into still larger units extending to the stage of insoluble particles, or perhaps such molecules may become attached to the various solid phase structures of the cells. Furthermore, it will be recalled from the discussion of electrophoresis that even the small ions may be extensively adsorbed on the charged surfaces of the large molecules and aggregates. It is even probable that uncharged substances like sugars may be partly immobilized by complex formation with the enzymes which utilize them as substrates. The net result of these considerations would be a marked reduction in the actual total concentration or osmotically effective concentration of independent particles and a lowering of the osmotic pressure of the internal phase of the bacterium. Plainly a turgor pressure should exist in a bacterium suspended in its normal medium, but the magnitude is still a matter of conjecture.

Up to this point the discussion has been limited mainly to systems made up of a solvent and solution separated by a membrane impermeable to

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prevents transfer of solvent by osmosis. However, the distortion or rupture of the wall depends upon the total force exerted on it rather than upon the pressure. Since this situation coincides in this case with small size, a wall, that would be considered weak if large, is capable of exerting high osmotic pressures.



the solute. It will be apparent to the reader that if the solvent is replaced by a solution containing the same solute or a different one still too large to pass the barrier membrane, osmosis will occur if the respective osmotic concentrations differ. Both solutions have characteristic osmotic pressures of their own when compared to the solvent, and as expected the attainment of equilibrium of solvent diffusion between the two solutions requires the application of a pressure equal to the difference between the two osmotic pressures measured against the solvent alone. For a closed system, such as a bacterium that is suspended in a medium, the net osmotic pressure will be less in a solution than it is in pure solvent.

With a system of the usual biological type which contains additional solutes that are small enough to pass through the differentially permeable membrane, a complication appears. The concentrations of such solutes will affect osmosis until the total particle concentration becomes equal on both sides of the membrane. While the solvent is diffusing so are the small solutes. This latter process is called *dialysis* and is slightly different from osmosis as usually defined and as treated in this discussion.

Dialysis is the process of the diffusion of solutes through membranes permeable to them and in no wise is concerned with movement of the solvent. This rather artificial distinction is useful because the dialysis of a solute is controlled only by the concentrations of that specific solute on the two sides of the membrane. In general, the presence of other solutes and their concentrations do not affect either the rate or equilibrium point of this process in dilute systems. (An exception called the Donnan equilibrium will be considered below). Recall that osmosis is controlled by total concentration. Hence, since the controlling factors and equilibria in osmosis and dialysis differ, it is convenient to employ different terms for the two processes.

### *The Donnan Equilibrium*

Electrostatic charges on particles that are too large to diffuse through a membrane will cause departures from ideal osmotic behavior. The equilibrium achieved under such conditions differs from that previously discussed and is called a *Donnan equilibrium*. This complication arises from the fact that the two solutions involved must each be electrically neutral, and the satisfaction of this condition requires that the concentration of dialyzable ions differ on opposite sides of the differentially permeable membrane. An example may serve both to illustrate and explain this phenomenon.

Let us assume the existence of a protein dissolved at a pH above its isoelectric point. The individual protein particles will then possess negative charges and in their diffusion will be accompanied by, but not firmly combined with, cations, e.g., sodium ions. This solution containing added so-

dium chloride is now separated from a solution containing only sodium chloride at the same concentration by means of a membrane permeable to the salt but not to the protein. Assuming that the pressures, temperatures, and volumes of the solutions are equal, what are the distributions of the ions present? The initial and equilibrium states may be represented respectively as follows in Figures 25 and 26:

I	II
$P^- = C_1$	$Na^+ = C_2$
$Na^+ = C_1 + C_2$	$Cl^- = C_2$
$Cl^- = C_2$	

FIG. 25. Initial conditions leading to a Donnan equilibrium.

$P^-$  represents the charged non-diffusible protein ion.

$C_1$  represents the equivalent concentration of protein and

$C_2$  molar concentration of sodium chloride.

I	II
$P^- = C_1$	$Na^+ = C_2 + x$
$Na^+ = C_1 + C_2 - x$	$Cl^- = C_2 + x$
$Cl^- = C_2 - x$	

FIG. 26. The same system at Donnan equilibrium;  $x$  represents moles of sodium chloride transferred.

In the original system (Figure 25) the sodium ion is in higher concentration in compartment I than in II and tends to diffuse into solution II. However, electrical neutrality must be maintained so anions must accompany the sodium. Since the protein cannot penetrate the membrane, chloride ions are carried along by electrostatic attraction. Chloride diffuses uphill as it were and sodium toward a lower concentration until these opposing tendencies counterbalance. Equilibrium is reached when the energy required to transfer a chloride ion equals that for a sodium ion, and the total free energy change thus equals zero. The free energy change required for the transfer of one mole of chloride from compartment I to II is:

$$\Delta F_{Cl^-} = RT \ln \frac{[Cl^-]_I}{[Cl^-]_{II}} \quad (1)$$

Similarly

$$\Delta F_{Na^+} = RT \ln \frac{[Na^+]_I}{[Na^+]_{II}} \quad (2)$$

At equilibrium

$$\Delta F_{\text{Cl}^-} + \Delta F_{\text{Na}^+} = 0$$

$$\therefore RT \ln \frac{[\text{Cl}^-]_{\text{I}}}{[\text{Cl}^-]_{\text{II}}} + RT \ln \frac{[\text{Na}^+]_{\text{I}}}{[\text{Na}^+]_{\text{II}}} = 0 \quad (3)$$

and

$$\frac{[\text{Cl}^-]_{\text{II}}}{[\text{Cl}^-]_{\text{I}}} = \frac{[\text{Na}^+]_{\text{I}}}{[\text{Na}^+]_{\text{II}}} \quad (4)$$

The ratio of the concentration of salt anion in compartment II to that in compartment I is equal at equilibrium to the ratio of the concentration of salt cation in compartment I to that in II. If these ratios are set up in terms of the concentrations at equilibrium, they may be evaluated on the basis of the initial concentrations of proteins and sodium chloride:

$$\frac{[\text{Cl}^-]_{\text{II}}}{[\text{Cl}^-]_{\text{I}}} = \frac{C_2 + x}{C_2 - x} = \frac{[\text{Na}^+]_{\text{I}}}{[\text{Na}^+]_{\text{II}}} = \frac{C_1 + C_2 - x}{C_2 + x} \quad (5)$$

Two equations containing two unknowns ( $x$  and a ratio) are thus written and  $x$  eliminated. As a result

$$\frac{[\text{Na}^+]_{\text{I}}}{[\text{Na}^+]_{\text{II}}} = 1 + \frac{C_1}{2C_2} = \frac{[\text{Cl}^-]_{\text{II}}}{[\text{Cl}^-]_{\text{I}}} \quad (6)$$

This relationship indicates that the equilibrium condition for the concentration of the diffusable ions depends upon the concentration of both the diffusable ions and the non-diffusable ion. It is clear from equation (6) that at a high equivalent concentration of protein the Donnan effect has a profound influence on the concentrations of diffusable ions. However, when the latter are relatively quite high the Donnan effect becomes negligible.

The Donnan equilibrium influences the osmotic pressure of a system containing non-diffusable ions since the osmotic pressure difference between the two compartments,  $P$ , should become

$$P = \frac{RT}{V} (\Sigma N_{\text{I}} - N_{\text{II}}) \quad (7)$$

where  $\Sigma N_{\text{I}}$  and  $\Sigma N_{\text{II}}$  represent the total numbers of moles of independent particles in compartments I and II respectively, and  $\Sigma N_{\text{I}}$  includes the protein. It may be calculated from equations (5) and (6) that the difference between the ionic concentrations, not including the protein, (called  $\Delta C$ ) in compartments I and II is

$$\Delta C = \frac{C_1^2}{4C_2 + C_1} \quad (8)$$



which suggests in connection with equation (7) that when the salt is low the osmotic pressure is higher than expected. When the salt is relatively high the Donnan effect becomes very small, and the osmotic pressure is close to that expected from the presence of uncharged protein alone. Equation (7) may be rewritten for convenience as

$$P = \frac{RT}{V} \left( C_1 + \frac{C_1^2}{4C_2 + C_1} \right) \quad (9)$$

Thus making clear the influence of the Donnan effect on the osmotic pressure.

It must be recognized that the distribution of hydrogen and hydroxyl ions as well as the ions of salts is affected by the presence of protein ions. Although this factor has been ignored in the foregoing discussion, a similar reasoning may be applied, and a more exact solution of the total Donnan effect may be obtained.

Before leaving this phenomenon let us briefly consider another consequence of the Donnan equilibrium. Owing to the uneven distribution of ions there must exist a potential difference between the two compartments. The equalization of this difference is prevented by the previously mentioned requirement for electrical neutrality in each compartment. The existence of the potential difference may be shown by inserting reversible electrodes into each compartment and connecting them externally. A current will flow through this external circuit, but electrical neutrality will still be maintained. This arrangement alters the ionic state, however, since ions are transferred to one compartment from its electrode, from that compartment across the membrane to the other compartment, and finally from there to the second electrode. The name *membrane potential* is applied to this potential difference arising as a consequence of the Donnan distribution of ions.

The reader will appreciate that the foregoing discussion is highly idealized by the presence of several assumptions. They include assumed ideal behavior of the solution, constant temperature and pressure, equal volumes in the two compartments, only one added electrolyte, and the absence of multivalent diffusible ions. Expressions may be derived to permit evaluation or incorporation of many of these factors when need arises, but the resulting equations often may be quite complex and difficult to apply in experimental practice.

### *Osmotic Phenomena Exhibited by Bacteria*

Fischer first demonstrated experimentally in 1897 that bacteria are subject to osmotic forces. He observed the plasmolysis of bacteria placed in

high concentrations of certain electrolytes, potassium nitrate being particularly useful.

*Plasmolysis* is the name applied to the shrinkage in volume accompanying the osmotic loss of water when cells are placed in hypertonic solutions. In the plasmolysis of bacteria the cytoplasmic membrane and cytoplasm shrink away from the more rigid cell wall, and the internal substance contracts to a single spherical or egg-shaped mass in the cocci and short rods. In the large rods the cytoplasm may contract and divide into separate granular masses. In strong solutions of salts the plasmolysis is rapid, but the cytoplasm may expand and again fill the cell after the salt has had time to dialyze into the cell. These results show that the organisms are not completely impermeable to salts but shrink only temporarily because of a rapid loss of water due to the relatively slow penetration of the salts.

*Plasmolysis*, the expansion in volume and bursting of bacteria placed in hypotonic solutions, was also first noted by Fischer. In plasmolysis the rigid cell wall is ruptured and the internal contents of the bacteria are partially extruded through the breaks. This process is brought about by osmosis leading to an internal pressure sufficient to overcome the structural strength of the organism.

### PERMEABILITY

The observations of plasmolysis and plasmolysis with bacteria suggest the presence of selectively permeable outer structures. The possibilities are these:

- 1) The cytoplasmic membrane acts as a semi-permeable structure.
- 2) Both the cell wall and cytoplasmic membrane are semi-permeable.
- 3) Only the cell wall acts as a semi-permeable membrane.

The observation of retraction of the cytoplasmic membrane from the cell wall during plasmolysis has been interpreted as pointing to the possession of properties of semi-permeability by the cytoplasmic membrane, therefore the third possibility has not received serious consideration. But this procedure does not resolve completely the role of the cell wall. As a fairly rigid structure, even if semi-permeable, it would not be expected that obvious shrinkage or expansion of the cell wall need occur in response to changes in the osmotic concentration of the environment. Nor can the fact that the cytoplasmic membrane responds to changes in osmotic concentrations when the cell wall shows no obviously distinguishable individual behavior be thought of as proving the lack of a role for the cell wall. The two structures conceivably might have different permeability properties. The increasing evidence for the structural complexity and the active metabolic role of the cell wall makes it highly unlikely that the cell wall should be non-selective.



tively permeable. A worthy contribution to knowledge would be made by studies specifically directed toward elucidating the nature of the permeability properties of the bacterial cell wall since no such studies are available for consideration at the present time.

Permeability is the sum of all the characteristics associated with the transfer of substances across membranes. The use in biology of the terms selectively permeable and semi-permeable membranes may actually imply both a qualitative difference, involving the absolute ability of substances to cross the cell membranes, and a quantitative difference relating to variations in rates of the transport of substances across membranes. More often than not, studies of permeability in biology refer to differences in the rate of penetration of substances. As is customary in physics and chemistry, it would be desirable to define permeability quantitatively in terms of an internationally accepted unit. Unfortunately, there is little agreement manifest in the literature dealing with problems of permeability. One practical value of a standard unit would be the facility lent to comparisons of permeability among different organisms or in different situations for the same organisms.

Permeability may be expressed quantitatively by means of a coefficient,  $h$ , stated in c.g.s. units:  $h = \frac{Q}{S(C_1 - C_2)}$ , where  $Q$  is the number of moles of substance diffusing per second across a membrane of area  $S$  in square centimeters when  $C_1$  and  $C_2$  are the concentrations of the substance in moles on both sides of the membrane. Such a quantitative formulation is a reminder that permeability is a function of time, surface area, and concentration gradient. Thus the observation that with a given solute concentration, a species of alga took two days to reach equilibrium, while the paracolon bacillus took only one minute might lead one astray in believing the bacterium to be more permeable. Actually, the differences in surface areas of the algae and the bacteria must be considered. The same data when calculated on the basis of the surface area involved give with these otherwise different organisms the same permeability coefficients, that is, the same magnitude of rate of transfer of solute across a unit area of membrane.

### *Methods of Studying Permeability*

Numerous methods for studying permeability have been used:

- 1) Visible changes within the cell, as for example, the penetration of colored substances.
- 2) Chemical methods. These depend on analysis for the transported substance. Either the uptake by the organism or changes in the amount of material in the external bathing fluid may be measured.



3) Isotope tracer methods. The uptake or changes in amounts of tracer isotope in the suspending medium are measured.

4) Electrical conductivity. This method is limited to studies of ion transport. When an electric current passes from a salt solution into a living cell movements of ions must take place. An increase in permeability to ions must result in a decrease in the electrical resistance of the organism. Therefore the measurement of the electrical conductivity of protoplasm may be considered to be a measure of its permeability to ions.

5) Electrical capacity. The greater the electrical capacity (the charge required to raise the electrical potential by a given amount) the greater the impermeability to ions. In conjunction with studies of electrical conductivity intelligent guesses may be made as to the proportion of surface available for ion transport. Thus an increase in electrical conductivity unaccompanied by significant changes of electrical capacity are interpreted as indicating only a small portion of the surface area of an organism to be permeable to ions. The electrical methods of measuring permeability are neither technically simple nor always theoretically satisfactory (see Cole, 1940). Studies with bacteria are not mentioned in review articles.

6) Osmotic methods. Since the gas laws have been shown to hold for osmotic phenomena, the osmotically induced changes in the volume of cells may be used as a measure of permeability. With cells having a rigid cell wall, it is desirable to start with a "normal" preparation and to measure the rate of volume decrease or plasmolysis. This method readily lends itself to quantitative expression in terms of a permeability coefficient (p.c.), useful for purposes of comparison:  $p.c. = \frac{c^1 - c}{c}$ , where  $c^1$  is a plasmolytic concentration and  $c$  is the concentration of the same substance theoretically isosmotic with a solution of a non-penetrating substance causing the same degree of plasmolysis. The time for recovery of a plasmolysed cell can also be used to measure the rate of penetration.

**INULIN SPACE METHOD.** In the methods of chemical analysis for quantitative measurement of the permeability of microorganisms such as bacteria and yeasts, it is desirable to have a means for determining intercellular space. When a mass of cells is added to a solution extraneous fluid (extracellular) is also being added along with the cells unless the cells have been thoroughly dried, usually an undesirable procedure. The fluid occupying the spaces between the cells dilutes the solute, and thus knowledge of its quantity may be a desirable datum in permeability studies. It is impossible to pack naturally shaped cells so as to eliminate all intercellular space. Only objects with the shape of an orthic-14 hedron could be packed so that all sides touch and intercellular space is totally eliminated.

To measure intercellular space, a known weight or number of cells is

added to a known volume of fluid. If the suspending fluid contains a solute it is obvious that the concentration of the solute (amount per unit volume) will decrease in direct proportion to the tendency of the solute to occupy the increased volume of liquid (solvent phase) arising from the addition of the mass of cells. Therefore, the measurement of intercellular space requires only knowledge of the decrease in concentration of some solute which *does not penetrate the cells*. The decrease in concentration of the solute is obviously directly related to the amount of intercellular space available for distribution of the solute. While several materials such as colloidal dyes, gelatin, and peptone have been used, the most satisfactory solute is probably inulin. Inulin is a non-toxic high molecular weight carbohydrate (3000) to which few organisms are permeable. Three per cent solutions are often used and changes in concentration are measured with satisfactory accuracy by both gravimetric and colorimetric methods. The essential concept of the inulin space method merely involves the realization that:

Total space = volume of original inulin solution + volume of mass of cells added.

Volume of mass of cells = volume of cells + intercellular space.

Space available for distribution of inulin = volume of original inulin solution + intercellular space.

Therefore, the ratio of the original concentration,  $C_o$ , of inulin before addition of the cells to the concentration,  $C_f$ , after mixing in the cells is an expression of the increased space occupied by the inulin which is the intercellular space. For example, if 1 unit of weight or volume of cells is added to 1 unit volume of inulin solution the intercellular space would be  $\frac{C_o}{C_f} - 1$  in terms of the volume and weight units employed. For general use the intercellular space in volumes per unit weight (or unit volume) of cells added may be expressed as

$$\frac{V_f - V_o}{W} = \frac{V_o}{W} \left( \frac{C_o}{C_f} - 1 \right) \quad (10)$$

where  $V_o$  is the volume of the initial inulin solution,  $V_f$  is the total volume of inulin solution after the addition of cells, and  $W$  is either the weight or volume of cells added. The intercellular volume per unit of cells corresponds to the lefthand side of equation (10) and may be evaluated from the direct measurement of the components on the right side of the equation.

Once the intercellular space is known the addition of a known quantity of a permeable substance to a given quantity of cell suspension should result in a calculable concentration assuming no penetration of the substance into the cells. The difference between this concentration and the actual



concentration found would represent penetration or uptake of the substance by the cells.

The extended discussion of the inulin space method has been presented because it is readily applicable to studies with bacteria, and bacteriologists should recognize its potentialities. Its extensive use might well help solve the problem of the permeability properties of the cell wall as distinct from those of the cytoplasmic membrane. By the means of this method Conway and associates have in a simple manner demonstrated with yeast that the cell wall has selective permeability properties and that these properties are not identical with the permeability of the remainder of the cell (the cytoplasmic membrane). Figure 27 outlines their procedure.

### ✓ *Theory of Permeability*

The movement of a substance through a membrane involves two sets of variables: the *permeability* of the membrane (the properties of a membrane which account for the penetration and passage of substances) and the driving forces of transport.

The forces responsible for the movement of a substance across a membrane are classifiable as different kinds of gradients: thermodynamic,<sup>4</sup> electrostatic, hydrostatic, and surface energy. The sum of the movements of a substance will be in the direction tending to reduce the gradient. At equilibrium the gradient will be reduced to zero, and there will be no further net transport. Thus if the cause of movement across the membrane is an activity gradient there will be no net transport when the activity of the substance on both sides of the membrane becomes equal since the rate of movement in both directions across the membrane will then be equal and constant. In biological systems such a true equilibrium is rarely found, there being more often attained a dynamic equilibrium or, as it is more commonly called, a steady state. Steady state equilibria have been thought of so frequently in connection with the most diverse kinds of biological phenomena that it is important for the student to be familiar with the concept. (See discussion p. 229.)

Permeability involving the aforementioned gradients has been labeled *permeation*. While permeation certainly occurs in biological systems it has become increasingly clear that it is not a sufficient explanation of permeability in all cases.

✓ Permeability by reason of *active transport* can also occur. Active transport is movement across a membrane requiring the expenditure of energy of metabolism. The exact way in which the metabolic energy is coupled to

<sup>4</sup> An activity (or effective concentration) or osmotic force due to difference in the concentrations on both sides of the membrane.



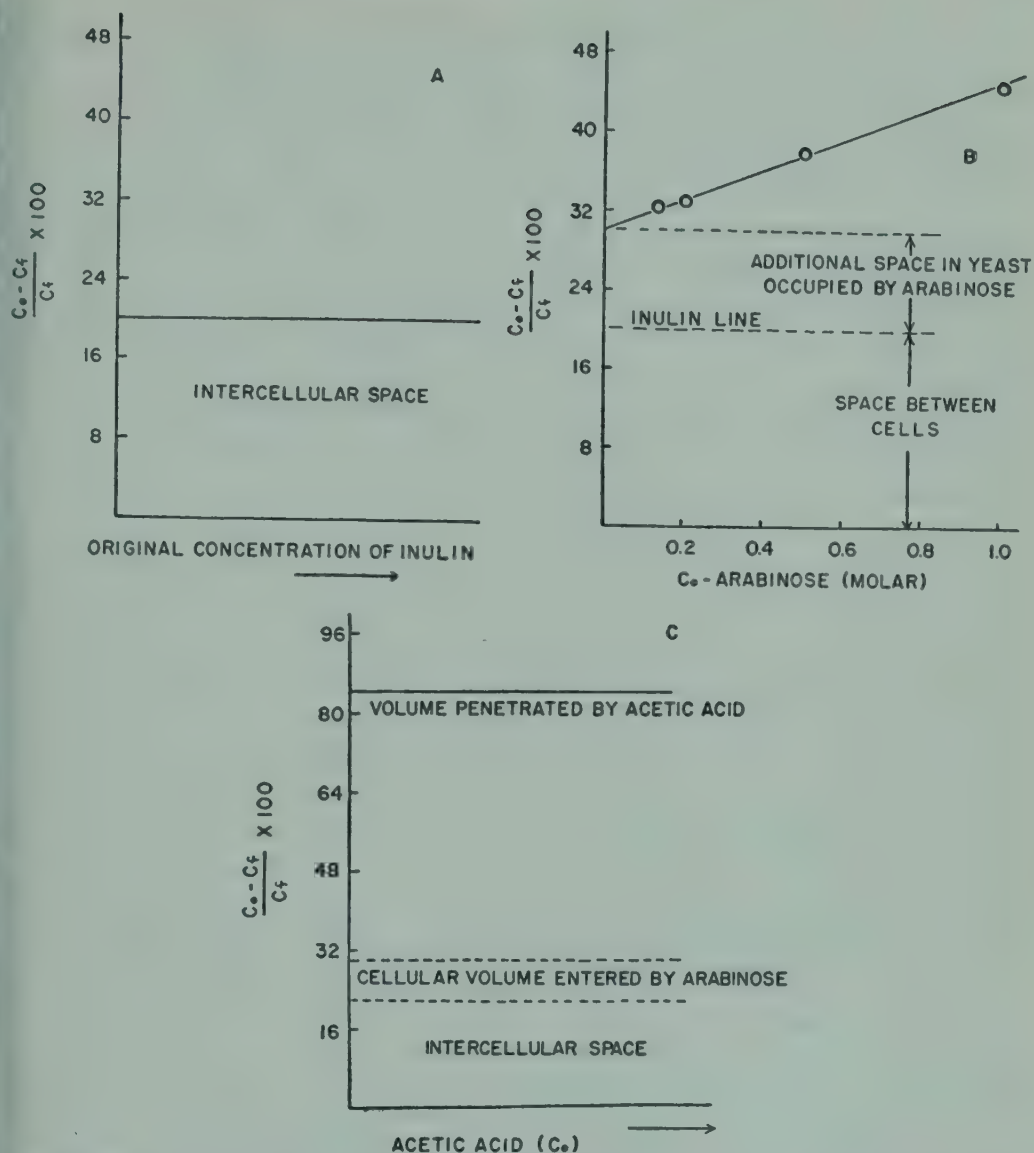


FIG. 27. Determination of the volume occupied by an outer selectively permeable region of cells. In baker's yeast this volume has been found to have the magnitude of the morphological cell wall.  $C_o$  is the original concentration of a test solute.  $C_f$  is the concentration of the solute after the addition of cells.  $((C_o - C_f)/C_f) \times 100$  is the percentage change in the concentration of solute after the addition of cells and is also an expression of the percentage of the volume of water in the mass of cells that is available for distribution of the solute. The position of the inulin line is found as described in the text and is indicated in diagram A. On the addition of varying concentrations of large solutes to a cell suspension the  $(C_o - C_f)/C_f$  values should not change and will correspond to the inulin space value if the solute cannot penetrate the cells and if the cells do not lose water or take water from the suspending medium. If a solute does penetrate, the difference in the  $(C_o - C_f)/C_f$  value for inulin and the solute being tested is a measure of the added volume or space being occupied by the test solute. This space is intracellular and indicates penetration of

the transport is unknown, though currently the subject of much speculation and interest. Active transport differs from permeation in the following ways:

- 1) It is associated with the expenditure of metabolic energy.
- 2) It makes possible the passage of materials otherwise unable to penetrate.
- 3) It can result in transport against concentration gradients not explicable by means of Donnan equilibria.

In the terminology of physical chemistry the membranes of organisms are separate phases existing in intimate contact with dissimilar phases on either side. The passage of substances by permeation across these membranes is a relatively complicated problem. For, unlike transport of free diffusion in homogeneous systems, the additional problems of diffusion across phase boundaries and the transfer of substances across the membranes between the phase boundaries must be considered.

The rate of passage of a substance across an interface depends on the rate at which the molecules of that substance strike the interface and on the probability of the molecules striking with sufficient force (possessing sufficient free energy) to cross the energy barrier presented by the interface. The free energy required for successful penetration can be considerably higher than that necessary for ordinary diffusion. In the case of ions studied in a model nitrobenzene-water system it was found that the free energy of activation required for diffusion across the interface was as much as three times that necessary for diffusion in the phases themselves. Ionic mobility

the cell. If this increased volume is less than the volume of the intracellular water contained by the cells, the solute cannot be occupying all of the space of the aqueous phase of the cell. While in the case of yeast, solutes have been found which seem to penetrate the whole cell as in diagram C, others such as galactose and arabinose seem to penetrate only a limited portion of the cell equal in magnitude to the space occupied by the morphological cell wall. Obviously yeast acts as though it possesses two barriers or membranes of different permeability properties. In plotting  $(C_i - C_f)/C_f$  against varying concentrations of  $C_f$ , if a horizontal line does not result, an osmotic concentration effect is indicated. A positive slope indicates movement of water into the cell and a negative slope a loss of water by the cell. Extrapolation to the ordinate permits estimation of the intracellular volume of water occupied by the solute and its relation to the inulin space and eliminates the complication in calculation introduced by the osmotic movement of water.

It should be emphasized that this procedure measures only volumes of aqueous phases. Hence the total volume available for penetration by ordinary solutes is less than the volume taken whenever solid phases are present. Thus acetic acid does not penetrate 100% of the cellular volume because not all of the cell acts as a solvent of this or any other polar solute. It will be apparent that this general procedure may be employed for a study of the quantity of available water in cells. During the discussion of "bound water", use of this solute distribution method was interpreted as disproving the existence of such restricted water.

(Adapted from Conway and Downey, 1950)

in this system was as much as a million times slower in the interface than in the water and nitrobenzene phases. One possible consequence of a high free energy requirement for transport across an interface may be that such diffusion could conceivably be a rate-controlling factor in permeability and also a rate-controlling step for subsequent metabolic processes.

It should also be clear that systems with high interfacial tensions are those which will require high free energies of activation for diffusion across the phase boundaries. Passage of polar substances would be easier across hydrophilic than hydrophobic interfaces and non-polar substances would pass more rapidly across the hydrophobic interfaces. Thus the degree of solubility of the penetrating substance in the phases would be expected to influence the rate of penetration through the phase boundaries. Experimentally this supposition is verified by findings with water-oil systems where the rates of diffusion across the interface prove to be inversely proportional to the water-oil partition coefficients of the solutes. ✓

Numerous mechanisms have been proposed to picture how substances cross biological membranes. Except for penetration by water the permeability of living organisms shows so remarkable a diversity that no single proposal is universally satisfactory. The most unrelated kinds of substances may penetrate or be excluded, non-polar and polar materials of all degrees of ionization and substances of varying molecular size from the smallest ion apparently to the high molecular weight proteins such as the extracellular enzymes and bacterial toxins. Permeability varies with the species, the physiological state and age of the organisms, the nature of the environment, and with the penetrating solute itself. In turn the influence of the solute on permeability is controlled by a number of factors:

- 1) Temperature. The temperature coefficients of permeability are positive and range from values close to unity to as high as 5. While temperature coefficients of 2 and higher are generally thought to be indicative of chemical reactions, permeability associated with high temperature coefficients may not always involve chemical reactions.

By way of explanation, let us consider the effect of temperature rise on processes like chemical reaction and diffusion. Temperature is, of course, a measure of the heat energy in a system and, therefore, a measure of the kinetic energies of the molecules. In any system there is an energy distribution with some molecules having much, others little, and the majority intermediate kinetic energies. If a process requires a considerable energy only the high energy molecules can react and the process is slow. When the temperature is raised, however, the energy distribution is shifted to a higher range, and the number of molecules possessing high kinetic energies is much increased, thereby greatly increasing the number available for reaction. In such a case the rate of reaction increases sharply with tempera-



ture and is said to have a high temperature coefficient as is characteristic of most chemical processes.

On the other hand, a process like free diffusion within a single phase involves all of the molecules present. True, the high energy molecules diffuse most rapidly but even the low energy molecules diffuse. Therefore, a temperature increase raises the rate of movement of all members, and diffusion speeds up as a function of temperature. However, since all molecules are involved anyway, the number able to undergo the process cannot increase, and the temperature change does not have such a profound influence as it does in the processes of chemical reaction which involve only the participation of the relatively few high energy molecules in the system.

Accordingly, diffusion is generally regarded as having a low temperature coefficient when compared with ordinary chemical reactions. However, when diffusion takes place across a phase boundary, this statement may no longer be true. To amplify this point, assume that high interfacial energies are present at the phase boundary. Such an energy screen may then hold in the given phase all but the fastest moving molecules which alone can pass across the energy barrier into the neighboring phase. Such a system will show a high temperature coefficient and differ in this respect from ordinary diffusion. It seems at present as though the high temperature coefficients of permeability might be accounted for in this way. In any case the occurrence of a high temperature coefficient cannot of itself be taken as sufficient proof of solute penetration of membranes by active transport.

In general high temperature coefficients have been found only for those solutes to which membranes are but slightly permeable. The other solute factors involved in permeability are the:

- 2) Solubility of solutes in the membrane.
- 3) Molecular volume of the penetrating solute.
- 4) Chemical reactivity of the solute with the substance of the membrane.
- 5) Relative polar character of the solute.
- 6) Kind and magnitude of the charge of ionizing groups of the solute.
- 7) The presence of other substances. Cases of both synergism and antagonism have been described. Generally it may be said that monovalent cations tend to increase permeability while polyvalent cations cause a decrease. In this latter capacity the divalent ions are quite effective, and the ions like ferric and aluminum decrease the permeability even more. The calcium ion seems to be a natural constituent of many biological membranes and to have an important role in maintaining the permeability properties under normal conditions of metabolism.

Membrane factors governing permeability are:

- 1) Presence and the capacity of colloids in the membrane for the sorption of penetrating substances.

- 2) Distribution and intensity of the electrical charge on the membrane.
- 3) The electrical potential gradient across the membrane.
- 4) Relative polar character of the membrane surface. ✓

✓ To account for transport across membranes the following hypotheses have been offered. Since these hypotheses are not known to be mutually exclusive it is logical to consider several of the proposals as having possible merit.

1) Sieve theory (Traube, 1899). The membrane is considered to be analogous to a sieve. The capacity of a substance to penetrate would be a function of the relative molecular volume of the penetrating substance and of the size of the "pores" of the membrane.

2) Selective solubility theories of L'Hermite and Overton. The solubility of substances in the membrane permits their penetration into the membrane and movement by free diffusion across the membrane. As long as the concentration of material on the two sides of the membrane is unequal, there should be a net transport in the appropriate direction. The lipid content of the membrane not only determines the permeability toward apolar substances but tends to predominate in characterizing the permeability properties of biological membranes. In harmony with this theory is the observation of increased permeability toward chemical substances in a homologous series as the lipid solubility increases despite increasing molecular volume. Thus organic acids seem to penetrate as the undissociated molecule since acetic, propionic, butyric and valeric acids though about the same strengths as acids are increasingly soluble in cellular lipid and penetrate most effectively in the order listed.

3) Adsorption theory (Tinker, 1916). Ions are thought to be adsorbed in accordance with classical quantitative laws and are released on the side of lower ionic concentration.

4) Reversible chemical combination theory. Substances react with components of the membrane to form unstable compounds or complexes. One variation of this last hypothesis is a process of exchange diffusion postulated for explaining the selective movement of ions across membranes. An ion species combines with a specific carrier within the membrane. Due to the selective solubility of the carrier in the membrane the carrier cannot leave the membrane, but by free diffusion it will transport the combined ions across the membrane. There will be a net movement across the membrane as long as the carrier is saturated with the combining ion and there is an unequal concentration of ion on both sides of the membrane. The carrier will release its ions to the side of lesser ion concentration and diffuse back toward the zone of high ion concentration.

5) Reversible phase transformation theory (Clowes, 1916). The membrane acts like an emulsion in which there is reversible change of internal



and external phases. Depending on circumstances the external phase at a given moment might be aqueous or lipoidal in nature. Net transport of substances of hydrophilic structure would depend on the presence of water as the external phase, and transport of apolar material would depend on an emulsion structure with water as the internal phase. Since the nature of the salts present is known to affect the structure of emulsions, this hypothesis might explain salt effects on permeability by their presumed action on the stability and nature of the disperse phase of the colloidal membrane. Calcium would favor a water in oil dispersion and sodium an oil in water dispersion. Antagonisms of ions would depend on their relative influence.

6) Mosaic structure hypothesis. It has been suggested that biological membranes are mosaics composed of portions having different selective permeability properties. The penetration of unlike substances whether based on their solubility in the membrane or other properties would then be a function of the presence and relative quantitative distribution of the appropriate, different areas of the mosaic. This hypothesis has been particularly attractive as a possible explanation for the fact that a given membrane can be selectively permeable simultaneously for both anions and cations. It has been possible to experimentally justify, but not prove, this theory with a model. In the model there is an actual physical barrier separating portions of the membrane permeable selectively to the anion and cation. The possible nature of the equivalent barrier in living systems is unknown.

7) Film penetration. An intriguing mechanism for the rapid transport of certain materials across cell membranes is suggested by studies of molecular films. Substances containing both polar and non-polar groups frequently may be spread as monomolecular films at liquid-air or liquid-liquid interfaces. Other materials capable of forming complexes<sup>5</sup> with the film do so when present, and if the mutual affinity involves both the polar and non-polar groups, the film becomes a monolayer of the complex. For example, when cetyl alcohol is spread on water, myristic acid injected beneath the film reacts with it. The reaction probably involves hydrogen bonding between the carboxyl and alcoholic hydroxyl groups. As a result two attached monolayers would exist and in many cases be stable. In this instance, however, the van der Waals attraction between the other groups of the molecules is large because both are large, non-polar chains. This additional force draws the side chains together yielding a monolayer with polar groups in the water and the non-polar groups in the less polar phase, in this case air. This process is called *film penetration*.

<sup>5</sup> A *complex* is a chemical compound resulting from the interaction of two individual compounds. In general the new bonds formed in complexes are weak and easily broken, readily yielding the original components.



It should be pointed out that materials forming complexes in a film will also form complexes in the bulk phase. Furthermore, materials penetrating a film but little may combine in a bulk phase with a third chemical species to yield a complex which then is able to penetrate the film. This observation has important implications in the action of drugs and toxic agents as well as in the transportation of metabolites and products.

In extending the concepts of complex formation and film penetration to membrane permeability the mosaic theory of membrane structure must be employed. It is postulated that discrete and adjoining areas of lipid and protein make up the cell membrane and that these zones extend in depth through this film. The second interface existing between the lipid and protein portions provides a potentially active path of transportation in the following way.

A surface active material that is also soluble in either the protein or the lipid phase will concentrate at the phase boundary. Since this concentration will greatly exceed that elsewhere in the system, it controls the rate of diffusion by providing a high concentration gradient. Thus the rapidly transported metabolite is visualized as penetrating the film between the lipid and protein layers, diffusing rapidly along the film and finally as passing to the internal phase of the cell as a result of the minimal concentration there. One would suspect that many materials not of themselves surface active but forming complexes with surface active compounds could then penetrate the interface and diffuse with otherwise unexpected rapidity.

All the hypotheses of penetration outlined can only explain how the movement of substances *might* take place across a membrane. It is reemphasized that the mechanisms suggested could result in a net transport only as long as a concentration gradient exists, but a consequence of such transport is an inevitable diminution and eventual disappearance of the gradient and the establishment of a true equilibrium. Even in the case of movement against a concentration gradient due to a Donnan equilibrium phenomenon, after the equilibrium concentrations of participating substances are reached, there will be no further net transport. In biological systems steady state equilibria are more probably established. In such systems the rate of transport of material in both directions across a membrane may not change with time, but there is a consistently greater movement in one direction than in the other. In cases not involving active transport and the expenditure of metabolic energy, this continuing transfer is probably due to binding of the material on one side of the membrane by normal metabolic processes. Substances carried across the membrane by permeation and delivered to the internal cellular structure may be precipitated, bound to inert structures, metabolically converted into new substances, or catabolized and eliminated as excretory end products.

Depending upon the hypothesis of permeability assumed, differing concepts of the chemistry and physical organization of protoplasmic membranes have been proposed. There is a more or less universal inclusion of both protein and lipid materials as essential constituents. A more recent theory, and an attractive one because it attempts to knit together diverse hypotheses of permeability, is the *tri-complex theory* of the structure of protoplasmic membranes. Protein, lipid-phosphatide, and a cation are presumed to be combined as a complex. As already noted a complex has been defined generally as an association product between two or more substances having physical and chemical characteristics different from those of either of the individual components. Often, but not always, it dissociates into its original components upon crystallization from solution. Complexes crystallizing from solution without dissociation tend to be labile, i.e., dissociate readily into their original components. The strengths of the bonds holding together the molecular components of the complex are usually rather less than those binding the atoms in typical chemical compounds.

The cation of the membrane tri-complex is thought to be calcium, but it can be exchanged for other ions with consequent changes in permeability and metabolism. Experiments with yeast have effectively demonstrated these results of cation exchange.

A virtue of the tri-complex theory is its ability to explain the divergent rates of penetration for various substances in different organisms. There would be unlimited opportunity for variation since the permeability will vary depending on the natures of the protein, the phosphatide, and the cation. Thus the nature of the protein would determine the ion exchange capacity of a membrane while the nature of the lipid-phosphatide with all the possible variations in the orientation of the lipid and phosphoric acid radical would be important in determining both the solubility of apolar compounds in the membrane and the membrane electrical potential. The latter would obviously also affect the transport of ions. The dissociation of the second hydrogen of the phosphoric acid radical is very sensitive to pH changes in the biological range and presumably could account for the cases of the sensitivity of permeability to pH changes. The tri-complex membrane also might act as a kinetically active surface with the chance drawing apart of molecules giving constant rise to temporary holes or pores through which molecules of other substances could slip as a result of their own kinetic or Brownian movement. This would help explain the cases where the sieve theory of permeability seems adequate.

### *Permeability of Bacteria*

Relative to the classical objects for permeability studies such as red blood corpuscles, eggs of marine animals and large-celled algae there have



been few investigations made using bacteria. What has been done indicates that the concepts of permeability developed from studies with other organisms are equally applicable to bacteria and that bacteria may present excellent examples for the study of special problems of permeability, particularly the problem of the coupling of exergonic metabolic reactions with the process of active transfer.

The data obtained with bacteria may be conveniently reviewed by considering the penetration of sodium, potassium, and amino acids each of which illustrates different aspects of bacterial permeability.

Penetration by sodium seems to be a typical case of permeation. Bacteria placed in hypertonic sodium chloride solutions in water or nutrient media are rapidly plasmolyzed and then deplasmolyzed. With *Escherichia coli* at room temperature, equilibrium is established in less than four minutes. By chemical analysis or measurement of uptake of radioactive sodium a direct proportionality can be shown to exist between the sodium concentration in the external environment and the rate of uptake, the final internal concentration being approximately equal to the external concentration. With *Escherichia coli* it has also been shown that the sodium uptake is independent of cellular metabolism and cannot take place against a concentration gradient. Washing bacilli with sodium-free fluid results in a rapid and fairly complete loss of the cellular sodium. All in all sodium seems to pass as an uncombined ion with equal ease in both directions across the cell boundaries and to exist in a free state within the bacterium.

The case involving potassium ion is considerably more complex. For while the potassium seems to move across the bacterial membranes by permeation, it can accumulate in the internal environment against a concentration gradient. There is no simple relation between concentration and uptake, and over a wide range of initial external concentration potassium is almost completely taken up (as high as 97%) by metabolically active cells. The accumulation of potassium against a concentration gradient is associated with the dissimulation of glucose. This correlation has been shown by noting the dependence of the accumulation upon fermentation and the inhibitory action of specific respiratory poisons such as sodium azide, 2,4-dinitrophenol, and iodoacetic acid. When bacteria are washed in isotonic potassium-free fluids there is little loss of accumulated potassium, but if potassium or its radioactive isotope is present there is rapid exchange between the internal and external potassium. In addition, hydrogen ions rapidly displace cellular potassium (Table 13).

With these facts in mind the penetration of potassium may be summarized as follows: The cell boundaries are highly permeable to the passage of potassium in both directions. This concept accounts for the rapid rate at which potassium is taken up and at which internal potassium exchanges



with external potassium. The movement against a concentration gradient and its association with glucose metabolism has been explained by assuming a binding of the potassium within the organism in the salts of hexosephosphates. The bound potassium is readily dissociated and could thus be displaced and rapidly exchanged with external potassium and hydrogen ions. However, the potassium hexosephosphates should be ionized, and the potassium ions should be available for exchange at all times for any cation capable of penetrating the cell. Since sodium ions do penetrate without displacing potassium some other mechanism for binding the latter ions must be operating.

Permeability toward amino acids is a still more complex case. Gram negative heterotrophic bacteria which do not require amino acids for growth are incapable of accumulating free amino acids as a part of their internal

TABLE 13  
*Displacement of  $K^+$  by  $H_2O^+$  in Aerobacter aerogenes*

pH OF BUFFER WASH FLUID	$K^+$ DISPLACED FROM CELLS* INTO MEDIUM
	%
7.1	9
6.64	48
5.66	63
4.3	79

\*  $190 \times 10^6$  organisms per ml.

(From Eddy and Hinshelwood, 1950.)

structure. In this regard they differ from gram positive organisms which can maintain a high internal concentration of a free amino acid against a concentration gradient. But the exact picture revealed with gram positive organisms varies with the particular amino acid. Thus while lysine penetration seems to be an example of permeation, the accumulation of glutamic acid involves active transport.

Permeation by lysine is indicated by the proportionality that exists between the external concentration of lysine and its rate of entry into the bacteria as well as by the slight effect of temperature on the rate of entry (a low temperature coefficient). It is also independent of exergonic metabolism. However, the free lysine which accumulates within gram positive bacteria does not diffuse out when suspended in distilled water unless glucose is also added. Since these observations are apparently in conflict they must be harmonized.

It has been noted that the rate of lysine accumulation increases with a rise in pH up to a value of 9.47, the isoelectric point for lysine. This observa-

tion provides the clue for an explanation of the leakage of free lysine from metabolizing cells placed in glucose solution that is consistent with a process of permeation. Lysine penetrates as the isoelectric molecule. But the internal pH of the bacterium is probably such that the isoelectric lysine changes into its cationic state once it has passed into the internal environment of the organism. The cell boundaries, while permeable to the isoelectric molecule, may be relatively impermeable to the cationic lysine. Thus the internal lysine will not leak out at an appreciable rate from the cell as long as the internal pH is low relative to the isoelectric point of lysine. When glucose is added and metabolized the internal pH may change to force more of the accumulated lysine into the isoelectric form, and this being able to penetrate the cell boundaries diffuses out into a lysine deficient medium at an appreciable rate.

With glutamic acid the permeability of the gram positive bacteria and yeasts seems to be a pure case of active transport. Placed in solutions of glutamic acid, such organisms do not permit penetration and accumulation of the amino acid unless glucose is present for oxidation. The temperature coefficient of accumulation is significantly higher than for the case with lysine. The rate of accumulation does not show any simple quantitative relation to the external concentration of glutamic acid and becomes maximal at about 50 micrograms per ml. with the organisms studied. The internal concentration of glutamic acid may be 50 to 60 times the external concentration. A number of inhibitors of glucose metabolism interfere with glutamic acid accumulation. On the other hand exposure of organisms to solutions free of glutamic acid does not result in leakage of free glutamic acid from the internal pool.

The permeability of bacteria may vary with their age, young cultures having the more permeable organisms, which is a reflection of a difference in their physiological states. But the organism may also mutate with respect to this property. This has been well established with *Staphylococcus aureus* and *Streptococcus faecalis*. It has also been suggested that the penetration of amino acids may not even be alike with all the individuals in a given clone of these species.

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## CHAPTER VIII

# Growth of Bacteria

Growth may be defined as the addition of new substance to individual cells or organisms. A decrease in size or in the quantity of substance of an organism has been spoken of as negative growth. The contrary phenomenon or the accretion of new substance at a greater rate than the loss of substance by catabolic metabolism is positive growth, and it is in this sense that the term growth is used generally. The addition of new substance may involve the mere movement of materials into organisms and either their incorporation as such as a part of the protoplasmic substance, as in the case of water and mineral salts, or their endothermic chemical transformation into new compounds as in the case of the conversion of amino acids to proteins. Thus growth is the sum of all the processes of catabolism and anabolism, and a complete understanding of the nature of growth is ultimately the study of the physiological basis of life. Nonetheless it is possible to study growth as the end result of metabolism without considering the intimate details of the intermediate metabolic processes leading to the addition of new substance to organisms. It is in this fashion that the growth of bacteria will be considered in the present chapter.

Growth is a process, a phenomenon which shows continuous change with time, and it should be studied quantitatively. Growth is characterized not only by the rate at which addition of new substance takes place but also by limits of absolute amounts of total substance which will accumulate under given conditions. Studied as a process, growth rates are measured, while as a scalar phenomenon, measurements of absolute quantities of substance are undertaken.

Growth is a phenomenon of the individual. The addition of new matter takes place in an organism. But the growth of an organism does not go on indefinitely. At a certain size the growth of an individual ceases, and with the unicellular organisms such as bacteria, the individual divides. The fact that in the same environment the resulting organisms can in turn grow and divide suggests that the original organism must have ceased growing as an individual not because of a limitation of environment but rather because of some internal or hereditary limitation. Properly, the study of biological growth is concerned with problems of the multiplication of the individual and the increase in numbers of individuals or populations.

Necessarily, then, the growth of bacteria will be considered as two broad

but not independent problems, the growth of the individual bacterium and the growth of populations or cultures. Inasmuch as the techniques for the measurement of changes in populations of bacteria are simpler than those available for the measurement of changes in the individual, the great preponderance of growth studies with bacteria have been studies of populations.

Growth requires the interaction of organisms with their environment and an active exchange of materials. Consequently the rates of growth should be governed by the laws of physics and chemistry determining the energy relations of the organism and its environment. The application of the laws of thermodynamics to growth phenomena would seem both a necessary and fruitful endeavor. It will therefore be desirable to briefly consider some recent thoughts on the problem of the application of thermodynamics to biological processes before proceeding with a specific discussion of the growth of bacteria.

### THE STEADY STATE AND BACTERIA

The laws of classical thermodynamics have been derived from studies of idealized closed systems.<sup>1</sup> Unfortunately the thermodynamics of closed systems does not apply generally to living organisms. In fact many of the properties of organisms differ so much from those of the usual closed, inanimate systems that some philosophers and experimentalists have proposed a vitalistic or non-material life force as responsible for growth, reproduction, death, and related phenomena. Most recently, however, the explanation of these complex processes has been attempted through the theory of steady states.

When materials flow continuously into a system, undergo a change, and flow out again in modified form, that system is said to be *open* and in a steady state. Although open systems present great difficulty to full understanding, they possess some important functional advantages over strictly closed systems, and it may be that these peculiar advantages permit the existence of life.

In a closed system a process can only proceed until the entropy<sup>2</sup> reaches

<sup>1</sup> The term "closed system" is applied to a material region in space that is isolated in all respects from all other regions.

<sup>2</sup> The entropy of a system is a measure of the disorder (randomness) of that system and increases as the disorder increases. Free energy may be regarded as the driving force of a process which will tend to take place when two components or two regions of a system differ in their free energies. A reaction occurring in a closed system will continue until the free energy difference between reactants and products has dropped to zero. At this time the reverse and forward reactions proceed at the same rate and no further net change occurs. The name equilibrium is given to this situation which represents the maximum disorder or entropy possible in the particular closed system.



a maximum value at which time the free energies of all parts of the system are the same with respect to the process in question. Work can be done by a reaction only while the entropy is increasing and free energy is being lost by one part of the system and gained by another. The entropy increase that always accompanies any spontaneous change is a fundamental characteristic of closed systems. In practice, many types of systems may be idealized for purposes of calculation as closed. However, natural systems are never strictly closed since heat transfer and the effects of measuring instruments are never completely eliminated. In many cases these factors may be made negligible but they affect the equilibrium state more or less so that there is really no existing closed system except perhaps the universe itself.

Many important systems do not even approximate the closed case, common illustrations being industrial plants and living organisms. These diverse examples have one thing in common, materials flow in and products flow out. Such systems are called open and have properties unlike those of closed systems. In fact, organisms differ so much from closed systems, and the supposedly immutable laws of the thermodynamics of closed systems fail so spectacularly with open systems that there have always been exponents of hypotheses postulating a unique life or vital force phenomenon as being responsible for all the unexpected properties of living organisms.

It will be clear that an organism plus its environment constitute a system that may be treated as closed. However, the organism itself cannot be so considered, and yet it warrants study as a separate, functioning unit. Recent efforts aimed at resolving this problem that show some promise are based upon extensions of generalized thermodynamic principles to include open systems. Such studies account for some of the characteristic properties of organisms and give promise of possible explanations of others.

As a cell does work on its environment the entropy of the cell may remain constant or actually decrease. This apparently anomalous behavior may be anticipated qualitatively from the generalized thermodynamics which states that an open system may decrease its total entropy while doing work because it is able to transfer entropy to the waste products of its activity. In other words, low entropy materials may be converted by an organism into high entropy materials without much effect on the entropy of the organism itself. It is necessary, however, that any working process in the total system of organism and environment will result in an increase in the total entropy. The foregoing statement leads logically to the observation that organisms function at the expense of their environment, the latter being finally degraded to a state of low free energy in which the organism can no longer function.

In open systems, time equilibrium either is absent or at most exists in



limited transactions of the system. Mathematical treatment is not entirely beyond us, fortunately, since the rate of transfer of reacting materials into the system is commonly either constant or periodic, and the products are continually or periodically withdrawn. When the transfers are continuous, the term steady state is applied. If the material transfers are entirely blocked, the system becomes closed, comes to equilibrium, and is *dead*.

Two important considerations have been put forth: (1) Since an organism is an open system, it acquires a greatly extended time scale because it does not come rapidly to equilibrium and cease functioning. Hence it has the consequent possibilities of development and of a maturity of approximate constancy. As the organism seriously depletes its environment, senescence and death should occur. (2) Organisms may increase their degrees of organization at the expense of disorganization produced in their environments. It seems possible from a consideration of the present theory of open systems that one direction of evolution might be toward organisms of more and more complex organization which are capable of utilizing more complex energy sources for more complex purposes. In other words, organisms ought to appear that are capable of entirely new functions.

Equations for the steady state may be solved with three interesting results. First, the solution indicates that the composition of an open system will remain constant even though equilibrium does not exist, reactions are actually occurring, and the reactions may even be essentially irreversible.

Next the proportion of the components in the steady state does not depend upon the environment and is governed only by the constants of the reactions in the system. In other words, the composition of a bacterium is independent of the nutrition within broad limits. Suspending the cell in distilled water will prevent growth, but the cellular components will remain essentially unchanged with respect to each other.

Finally, an external disturbance or stimulus will tend to produce an opposing response. For instance, if the rate of catabolism be increased, metabolites in the cell will tend to decrease. However, since these metabolites diffuse into the cell as a result of concentration gradients and since these gradients become correspondingly increased as the metabolite concentration in the cell decreases, the rate of diffusion increases and the supply of metabolites is kept high. We, therefore, describe the cell and open systems in general as being adaptable.

A resting cell, although performing no perceptible work, apparently requires a continuous supply of energy for maintenance of life. If an aerobic organism is deprived of oxygen, life ceases, or if an anaerobe is in any way prevented from using other compounds for basic oxidations, it dies. The cell needs energy in applying pressure to prevent unlimited os-

mosis, it needs energy to maintain ion concentrations different from those in the environment, and it needs energy to preserve high concentrations of high energy materials. For example, the equilibrium between amino acids and proteins in the presence of catalysts is far toward the amino acids, so that a high concentration of proteins can exist only when energy is continuously supplied. The energy thus required by the steady state may account for the comparatively low efficiency of living organisms which are never able to transform more than a fraction of the available free energy into effective work. If these energy considerations are correct, heat should be evolved on passing from the steady state to equilibrium when the cell dies. Unfortunately, this point has not been settled, different investigators reporting contradictory results.

Various workers have used the concepts of open systems and steady states in their efforts to account for biological phenomena. Among these applications we may include metabolism as maintenance in a steady state. The organism may be randomly disturbed, but after each disturbance it returns to the steady state. Periodic fluctuations are recognized repeating processes superimposed on the general steady state and being periodic may themselves be regarded as a sort of steady state. Growth, development, maturity, senescence and death are respectively approaches to the steady state, the steady state itself, and finally a slow drifting toward equilibrium. A theoretical model of a cell has been deduced for which an average typical cell size may be predicted and for which growth and periodic division would be expected. Elementary self-multiplying biological units, genes, chromosomes, and viruses may be treated as metabolizing aperiodic crystals by assuming that degradative processes are occurring in the system leading to repulsive forces which ultimately increase to critical values and cause division.

In addition, the well-known phenomenon of the selective accumulation of salts and metabolites against concentration gradients has been studied and yields mathematically to the theory of open systems. Finally, a quantitative theory of growth itself has been developed and is discussed by von Bertalanffy:

"Growth is considered to be the result of the counteraction of anabolism and catabolism of the building materials. By quantitative expressions, using the physiological values of anabolism and catabolism and their size dependence, an explanation of growth in its general course, as well as in its details, and quantitative growth laws have been established. This theory . . . permits precise quantitative predictions which have been verified . . . by later experiments. The conceptions of dynamic morphology have been applied to . . . the quantitative analysis of growth in microorganisms, invertebrates, and vertebrates, the physiological connections between me-



tabolism and growth, leading to establishment of metabolic types and corresponding growth types, allometry, growth gradients and physiological gradients, pharmaco-dynamic action, and phylogenetic problems."

### GROWTH OF THE BACTERIUM

Theoretically the deposition of new substance in a growing bacterium could take place along any or all of the three possible dimensions of space and result in increases in length, width, and depth. Ignoring external forces of deformation the shape of any organism is inevitably predetermined by the relative rates of growth along these dimensions. As has been pointed out previously, objects as small as bacteria would tend to be spherical in shape as a result of interfacial tensions if they did not possess a mechanically rigid cell wall. The relatively fluid internal protoplasm of the bacterial cell, if unconfined by the rigid cell wall, would tend to assume a spherical shape independent of the relative rate of deposition of new substance along the possible geometric axes. Thus in spite of the general principle stated, the fact that growing bacteria maintain other than a spherical shape is no necessary proof of a disproportionate deposition of cytoplasmic substance along the geometric axes but rather it might be attributed primarily to the unknown ways in which new additions are made to the dimensions of the rigid cell wall.

Bacteria are only infrequently spherical. Even in cocci, growth cannot be equal in all dimensions for it has been shown in the case of *Streptococcus faecalis* that the shape of the coccus may be more accurately described not as a sphere but as a flattened ellipsoid of revolution which during growth slowly becomes an elongated ellipsoid. The transition is marked by the only temporary assumption of a spherical shape. The mathematical expression of the form of bacteria has been thoroughly summarized by Knaysi (1951). Such formulations are useful particularly when data are desired which may be plotted for the graphical representation of changes in shape accompanying growth.

Though the direct measurement of the growth of individual bacteria has been reported for relatively few species, differences in the nature of the rate of increase in size have been found. A sigmoid or S-shaped curve for growth is typical of the cells of higher plants and animals and microbes other than bacteria including both budding and fission yeasts. In *Streptococcus faecalis* the rate of growth follows such a sigmoid growth curve, growth being least just prior to and after division, and maximal when the organism is about three quarters its adult size. The term adult size is used for a bacterium to signify the size of the organism at the time of its division. In contrast is the unexpected finding of a relatively constant growth rate



throughout the individual life span of some multiplying aerobic spore-forming bacteria and the gram negative *Enterobacteriaceae* (fig. 28).

A plausible hypothesis has been presented suggesting that the sigmoid growth curve is characteristic only of uninucleated cells. Active growth in

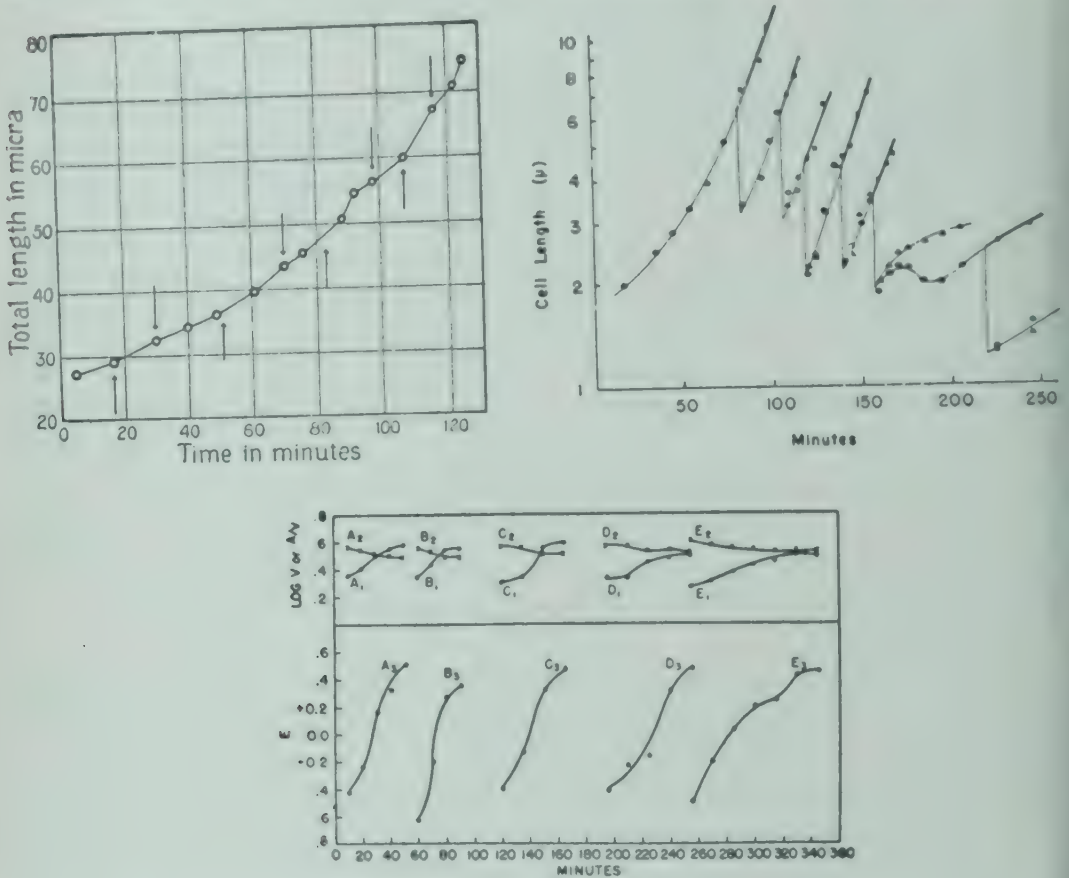


FIG. 28

(a) Upper left. Growth in length of a chain of *Bacillus ramosus*. Arrows indicate times at which fission was noted (Redrawn by Adolph, 1931, from data by Ward, 1902).

(b) Upper right. Increase in length of growing *Escherichia coli*. Vertical lines indicate new individuals resulting from fission. From the upper ends of these vertical lines the growth curves are double, representing the sum of the lengths of the two daughter cells formed. Note that there is no break in the growth rate at the time of fission and that the growth rate is logarithmic for the first few generations.

(Adapted from Bayne-Jones and Adolph, 1932)

(c) Lower. Growth of *Streptococcus faecalis*. Curves A<sub>1</sub> to E<sub>1</sub> represent growth in volume of individual organisms, and the sigmoid shape should be noted. A<sub>2</sub> to E<sub>2</sub> represent the surface area-volume ratio during growth of the same organisms. Curves A<sub>3</sub> to E<sub>3</sub> represent the changes in form *E* of the organisms. *E* is equal to  $1 - \frac{b^2}{a^2}$  when *b* is half the maximum width and *a* half maximum length of the organism; thus at *E* = 0 the organism is a sphere.

(From Knaysi, 1951)

cell size occurs during the so-called resting stage of nuclear activity and slows down during mitosis. The mitoses or nuclear divisions of multinuclear cells need not occur all at one time nor coincide with cell division, so that the rate of growth of such cells could be relatively independent of the stages of nuclear division and fairly constant as long as some of the nuclei in the growing synctium were in a resting stage. The differences noted in the growth of bacteria therefore have been postulated to be indicative of a difference in the number of nuclei present per bacterium, those having a sigmoid curve of growth being uninucleate organisms and those with a different growth curve being multinucleate.

✓ The increase in size of bacteria during growth is influenced by hereditary characteristics and environmental factors. Certainly heredity must determine how the organism will respond to environmental influences and must be responsible for the differences among species subject to the same environment. The increase in bulk of a bacterium is accompanied by an increased uptake of water as well as synthesis of organic matter. In actively growing *Proteus vulgaris* a five-fold increase in volume was found to be accompanied by only a two-fold increase in dry weight. This indicates water uptake to be chiefly responsible for the increase in bulk. Though water uptake relative to dry weight increase is generally not as disproportionate as in this reported case of *Proteus vulgaris* the same general situation has been observed with other bacteria. ✓

The actual increase in size of individual cells must be a reflection of the ratio existing between the rate of accretion of new substance and the rate of fission, and while division is dependent on growth it can vary independently of growth. For this reason the absolute size of cells is not fixed, a necessary condition if the mechanisms of growth and fission were identical or varied in exactly the same manner. Evidently there must exist special mechanisms of fission with characteristics of their own apart from those of growth.

As a rule the mean adult size of most unicellular organisms is greatest at the lower temperature limits of growth. Such an observation suggests a lower temperature coefficient for growth of the individual than for multiplication. An exception is the reported case of the yeast *Saccharomyces cerevisiae* showing a similar size over the fairly wide range of 21 to 37°C. For the bacteria there are numerous data available relating temperature to fission rates and population densities. But unfortunately practically no data exist on the differences in mean adult size of the same species at different temperatures and none are reported on the possible effects of temperature on the shape of the growth curve of individual bacteria. ✓

Figure 29 illustrates a typical case of how the multiplication rate of rod-shaped bacteria increases with temperature. Unfortunately no simultaneous record was made of the growth rate so that these data cannot be used to

provide information of the effect of temperature on growth and thus the volume of adult bacilli. Numerous authors report an increasing fission rate with increasing temperature up to some limiting temperature of growth. At temperatures above that supporting the most rapid division rates there is a short temperature range over which there is commonly exhibited a precipitous drop in fission rate. Microscopic examinations of cultures at these higher temperatures often reveal living bacteria of bizarre shapes and of great size. Such observations are explained by the greater sensitivity of fission than growth to the damaging effects of excessive heat. Obviously the increased size of the bacteria is due to a greater reduction of the fission rates than of the growth rate, but such temperatures actually are harmful

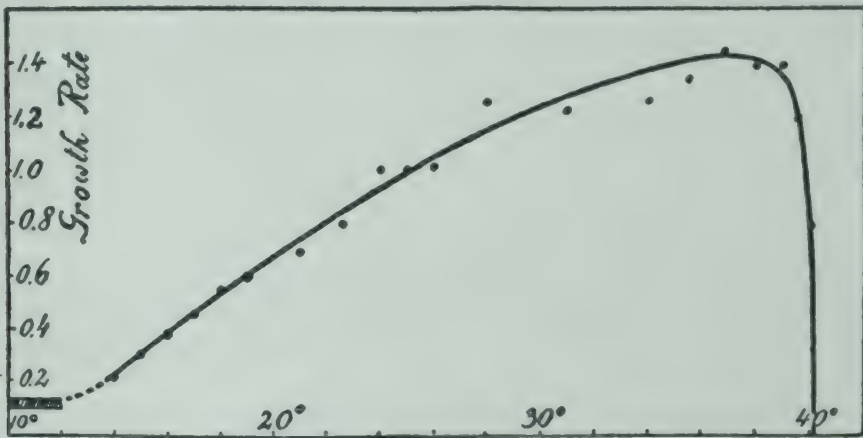


FIG. 29. Multiplication rate of *Bacillus ramosus* at different temperatures computed as the growth rate constant of Slator.

(Rahn, 1932, calculated from data of Ward, 1902)

for both division and growth as indicated by a decrease in the total mass of organisms per unit volume of culture.

Zoologists have long recognized Bergmann's rule which states that the body sizes of races living in cooler climates are larger than those of races of the same species living in warmer zones. This rule has been found applicable to both vertebrates and invertebrates. A similar kind of situation seems evident among the aerobic spore-forming bacilli, a genus marked by great differences in the adult sizes of different species. The larger species of the genus have lower temperature growth ranges than the small-celled species (Table 14). Unfortunately no similar reports are available for other genera of bacteria.

Since the regulation of size is a characteristic of living forms, the question has been asked why the growth of an individual cannot proceed indefinitely in an apparently favorable environment? Or as the question may be framed for bacteria: What determines the point in the growth of a bacterium at



which division takes place? Answers to these questions still remain chiefly in the realm of speculation. The classical hypothesis has been the Leuckart-Spencer principle which states that as the organized unicellular body increases in bulk its surface becomes proportionately less and leads to the

TABLE 14

*Temperature growth ranges and cell sizes of some members of the genus Bacillus*

SPECIES	NO. OF CULTURES	TEMPERATURE		CELL SIZE IN MICRA		
		Minimum	Maximum	Width	Length	Median
		°C	°C			
<i>Bacillus subtilis</i>	16	15	55-60	0.47-0.83	1.77-2.77	0.62 × 2.27
<i>Bacillus vulgatus</i>	3	10-15	55-60	0.46-0.66	2.16-2.50	0.56 × 2.33
	9	10-15	50-55	0.46-0.98	1.69-3.95	0.68 × 2.80
<i>Bacillus mesentericus</i>	2	10-15	55-60	0.62	2.00-3.08	0.62 × 2.40
	5	10-15	50-55	0.58-1.00	1.52-3.08	0.70 × 2.40
<i>Bacillus agri</i>	5	15-20	50-60	0.46-0.58	2.62-3.32	0.53 × 2.45
<i>Bacillus cereus</i>	20	10-15	40-50	0.70-1.41	3.15-5.85	1.00 × 3.60
	11	5-10	40-50			

(From Lamanna, 1940.)

TABLE 15

*Relation of volume to surface of a cube*

EDGE OF CUBE	DIMENSIONS OF CUBES									
	1	2	3	4	5	6	7	8	9	10
Surface.....	6	24	54	96	150	216	294	384	486	600
Volume.....	1	8	27	64	125	216	343	512	729	1000
Surface.....	6	3	2	1.5	1.2	1	1	1	1	1
Volume.....	1	1	1	1	1	1	1.16	1.33	1.5	1.66

necessity for segmentation or subdivision of the organism. As a statement of a law of geometry this hypothesis is secure (Table 15). It is less evident as a proper explanation of the limitation of growth of individual organisms. In the case of bacteria there is actually evidence which may be adduced against this hypothesis.

The reason presumed for the existence of a critical ratio of surface area to volume as a determinant for fission would be related to the capacity of essential nutrients to be transported at the necessary rates to internal body

regions of consumption and assimilation. With any increase in cell volume without a proportional increase in surface area the likelihood of an insufficient supply reaching internal areas would increase and growth must be reduced or cease. The same consideration applies to the diffusion of toxic metabolic products from the cell if growth were self-limiting because of the internal accumulation of harmful waste products. For a given external concentration of food it is possible to calculate the shortest radius of an organism at which the concentration of the nutrient within the organism would drop to zero. This radius for an ellipsoid is equal to  $\sqrt{\frac{5D \cdot C}{A}}$  where

$D$  is the rate of diffusion,  $C$  the concentration at the surface, and  $A$  the rate of consumption of the food. For objects as small as bacteria it has not been shown that this limiting radius approaches the dimensions of actual bacteria. It is also evident that this limiting radius should increase in a mathematically definable way with an increase in the external concentration of food. Thus, if the Leuckart-Spencer hypothesis holds, other conditions being equal, the mean adult size should be greater in richer culture media. The applicable data with bacteria are not consistent. A proportional increase in size of *Bacillus megaterium* with increase in nutrient concentration has been reported. However, with a diphtheroid organism Henrici (1928) found great variation in size on increasing the nutrient concentration with a preponderant number of smaller-sized organisms.

Of even greater interest is the knowledge that with the cylindrical, rod-shaped bacteria an increase in volume during growth occurs most often without any significant change in the surface area-volume ratio. This situation arises because these cylindrical bacilli grow chiefly in length and rather little in width.<sup>3</sup> Of all the dimensions of rod-shaped bacteria at any one cultural age the volume rather than any other spatial dimension varies least at the onset of division. Furthermore, with *Escherichia coli* the longest individuals in a culture were found in the early phases of the culture's development to be also the thinnest bacilli but as the breadth of the multiplying bacilli became more nearly average as the culture aged then the lengths also diminished. Thus changes in shape of dividing organisms tended to occur with no change in volume rather than in surface area-volume ratio. These findings may indicate a critical volume of the individual as controlling the onset of fission in some unknown manner. In any case they do not support the concept of control exercised by a critical ratio of surface area to volume.

<sup>3</sup> The volume of a cylinder =  $\pi r^2 h$  and the surface area =  $2 \pi r h$ , thus if  $h$  (length of bacillus) increases during growth with no significant change in  $r$  (radius of bacillus) the increases in both volume and surface area are directly proportional to the increase in  $h$  and the ratio of surface to volume is unchanged.



Finally, the nature of the growth curve of individual bacteria may be considered as an argument against the surface area-volume ratio hypothesis. The surface area-volume ratio is greatest immediately after division when the newly formed organisms are the smallest size and would decrease progressively as the organism grew to the adult size. Thus according to the Leuckart-Spencer principle the growth rate might be expected to be greatest at the birth of the bacillus when the surface area-volume ratio is most favorable and progressively decrease to the time of division. The growth curves of individual bacteria, while varied, certainly do not correspond to this hypothesized sequence of changes.

Increasingly, biologists characterize the nucleus as more than a passive carrier of the hereditary units. The hereditary mold of the body is viewed as being fashioned by the intimate participation of the resting nucleus in the synthesis of cellular components, particularly protein. The active role of the nucleus in the growth process may lend support to an old hypothesis (Hertwig, 1903) which states that a nucleoplasmic index exists which is a determining factor in cell division. The nucleoplasmic index would be equal to  $\frac{V_n}{V_c - V_n}$  when  $V_c$  is the volume of the cell and  $V_n$  the volume of the nucleus at the stage of cell division. The characteristic index would have to be determined empirically for each species. Since the cytoplasmic volume increases during the resting stage of the nucleus without a proportionate increase in nuclear volume this index would tend to be exceeded during growth. Consequently, in unicellular organisms the index would be maintained by the cessation of cytoplasmic growth and the division of the cell and nucleus. The present inadequacies in the knowledge of bacterial cytology do not permit an evaluation of this concept for the bacteria.

## DEVELOPMENT OF BACTERIAL CULTURES

### METHODS

The development of a bacterial culture is accompanied both by an increase in the quantity of protoplasm and in the numbers of organisms. Since no constant relation exists in the ratio of increase in protoplasmic mass to rate of bacterial multiplication, it is necessary to distinguish between these phenomena. Some authors in the past have not always clearly distinguished in their writings between growth and multiplication. In the present case we have used, and will continue to use, the term "growth" to signify the addition of new substance to protoplasm or a net increase in protoplasmic bulk. Increase in population or the multiplication and increase in number of individuals will be referred to as such or by other unambiguous and self-evident terms.

No single method in use permits the simultaneous quantitative estima-



tion of both protoplasmic mass and numbers of bacteria. Fortunately, these quantities may be related by comparison of data obtained by different methods on one sample or on representative aliquots of that sample. Once the relationship between methods is established for given strains of bacteria

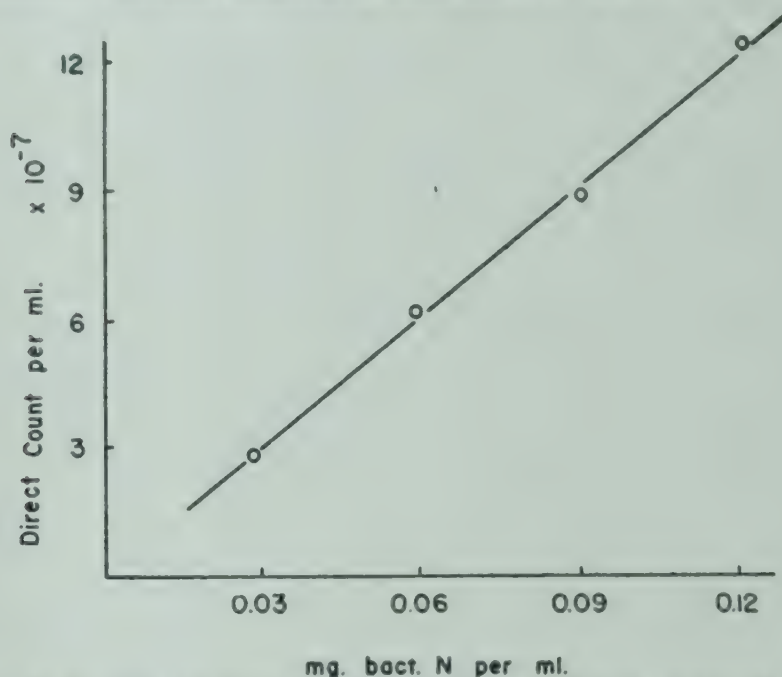


FIG. 30. Relation of the number of bacteria to the mass of bacterial protoplasm. Any satisfactory methods for obtaining the required data may be employed. In the example shown the number of *Clostridium botulinum* organisms obtained by a direct microscopic count has been plotted on the ordinate and the mass of bacterial protoplasm as bacterial nitrogen has been plotted on the abscissa. Thus with this same organism under conditions similar to those with which the data were obtained one method alone can serve to estimate bacterial nitrogen and numbers of organisms by referring to this curve. Such a curve may be called a "reference" or "standard" curve. Not all the methods of the bacteriologist will show as nearly a linear relation as illustrated in this graph. It is therefore necessary in preparing a standard curve to be sure to obtain data over the actual range of quantities to be measured. It is not safe to assume a linear relation and to plot merely two points on the graph and connect them by a straight line.

the two quantities may be estimated by one method alone, provided the conditions of culture are not changed. Ordinarily this calibration is done by plotting the various data obtained as a reference curve, the quantity not directly measured in subsequent observations being estimated by interpolation. Figure 30 illustrates the principle. Irrespective of their major interest, investigators who wish to obtain the greatest return for their investment of effort and time will always work out such standard curves. In unanticipated ways it may be of great value to know whether the var-

ables under study do or do not affect growth and multiplication in the same way.

Methods for estimating growth or increase in amount of protoplasm may be briefly listed as follows:

(1) Direct. These involve measurement of some parameter of volume of protoplasm or some constituent of it.

a. Centrifugation. Under specified conditions the bacteria in a given volume of culture are centrifuged, usually in a capillary tube. The height of the packed organisms is a measure of the total protoplasmic mass. If the specific gravity or average size of the bacilli are known the number of organisms may be calculated. (See Schmidt, 1926, and Schmidt and Fischer 1930.)

b. Dry weight. The weight of dried organisms per unit volume of culture may be determined. This method ignores the water content and its variation during the growth of bacilli, but it is a more satisfactory measure of protoplasmic mass than wet weight. Determinations of wet weights are not precise nor accurate because of the difficulty of evaluating the relative contributions of water wetting the bacterial surface and intracellular water. Dry weight determinations of bacterial protoplasm are subject to the same difficulties ordinarily associated with the estimation of the dry weight of proteins.

c. Nitrogen. Cells are separated from known volumes of culture, washed to remove the nitrogenous constituents of the media, and nitrogen determined by a micro-Kjeldahl procedure.

d. Colorimetric or spectrophotometric estimation of a constituent of protoplasm. Appropriate quantities of washed bacilli from known volumes of culture are hydrolyzed or otherwise treated to permit the release of the characteristic organic constituents of protoplasm. These products may be treated with reagents yielding colored products, and the original quantity of protoplasm estimated from the intensity of the developed color. An example is the determination of tyrosine and tryptophane by the Folin-Ciocalteu method. Compounds with characteristic absorption spectra may be determined spectrophotometrically as is often done for purine and pyrimidine derivatives by measuring absorbancy at 258 m $\mu$ .

(2) Indirect.

a. Measurement of the consumption of a metabolite or accumulation of a metabolic product. Oxygen consumption and the production of acid from a fermentable carbohydrate have been used to estimate bacterial protoplasm. These methods may be satisfactory when nothing is operating to limit oxygen consumption or acid production apart from coincidental effects on growth.

b. Turbidimetry. Bacteria are opaque and in suspension will exhibit a Tyndall effect similar to any colloidal system. Therefore, either the optical density or light scattered by bacterial cultures may be employed to measure the amount of bacterial protoplasm. A most simple method has been to determine the depth at which a wire loop plunged down into a bacterial suspension disappears from view by the naked eye. A comparison of turbidity may be made with standard suspensions of colloidal particles as with the MacFarland nephelometer (Table 16). However, the recent tendency has been to use commercially available photoelectric devices to estimate opacity.

TABLE 16

*Preparation of a MacFarland nephelometer, namely a series of standard tubes of varying turbidity*

TUBE.....	1	2	3	4	5	6	7	8	9	10
ml. BaCl <sub>2</sub> (0.1%).....	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
ml. H <sub>2</sub> SO <sub>4</sub> (1%).....	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9.0

Methods for estimating the multiplication or the numbers of organisms may be briefly listed as follows:

(1) Microscopic or direct methods. Unless differential or vital staining procedures are used these methods count both dead and living organisms without distinguishing between them.

a. Counting chamber. Commercially available counting chambers such as the Petroff-Hauser or Helber bacteria counters are employed. The principle of their use is to determine the number of bacteria in the fluid volume above calibrated ruled areas etched into glass slides. Since the depth of fluid above the ruled area is known the average number of organisms per unit volume may be calculated. In the Petroff-Hauser counter the depth of fluid is 0.02 mm. and the ruled areas are 1,400 square mm. Each bacterium in the volume of fluid above one square thus represents 20,000,000 organisms per ml. of culture. Obviously a disadvantage of these counting chambers is that they can be used only with fairly large numbers of organisms.

b. Stained smears. A known volume of culture is spread over a measured area on a slide. The slide is then fixed and stained, and using a microscope whose field diameter for a particular combination of objective and ocular is known, the concentration of bacteria may be calculated from the average number of organisms per microscopic field. The equation for this calculation is:



$$\text{Cells per ml. of culture} = \left( \frac{\text{area of smear}}{\text{area of microscopic field}} \right) \times \left( \frac{1 \text{ ml.}}{\text{volume of sample}} \right) \times \left( \frac{\text{average no. of bacilli per field}}{\text{volume of sample}} \right)$$

c. Proportional count, Wright (1902), or Fries (1921) method. In this procedure equal volumes of a standard suspension of yeast cells, blood corpuscles, or inert particles and the bacterial culture are mixed. By microscopic examination the ratio between the bacteria and the particles is determined, and the number of bacteria per ml. of culture computed. Since only a ratio must be determined it is not necessary to count definite volumes, and the material may be counted in wet or dry preparations.

(2) Indirect methods. These depend on the capacity of organisms to grow out under the conditions of the test when transferred to new media. As a result such methods count only living cells and not always all of these. Consequently they give estimates of bacterial numbers consistently lower than the microscopic methods of enumeration.

a. Dilution or most probable number method (McCrary, 1918). The culture is diluted out to the point where the diluted samples when seeded into fresh media do not yield growth. Assuming that the organisms are distributed randomly in the diluted samples and that any viable organisms present in these samples will grow out in the new medium, the original density of the culture's population may be estimated by the application of the theory of probability (see Am. Pub. Health Assoc., Standard Methods for the Examination of Water and Sewage, 1941; Halvorson and Ziegler, 1933; Cochrane, 1950). The precision of the method is dependent directly upon the number of samples taken per dilution. But even with large numbers of samples the precision remains low and is significantly poorer than plating methods.

### (3) Plating methods.

a. Roll tube. A melted agar medium and dilutions of cultures are mixed and rotated in a sterile test tube, held in a horizontal position. The medium will solidify on the walls of the tube. The tubes are then incubated, and the resulting colonies counted.

b. A mixture is made of a melted agar medium and a sample of culture in a petri dish.

c. Surface colony count. A petri dish containing a solidified agar medium is inoculated by either spreading a sample of culture on the entire surface or by depositing a small sample on the surface as small drops with a calibrated capillary pipette (Miles, Misra, and Irwin, 1938). These surface colony counting methods seem to give the best replication in counts between plates.

Of all the methods listed, turbidimetry and plate counting have had the greatest use. This usage is due to their relative simplicity and a precision satisfactory for most purposes. A critical study of the errors inherent in plate counting methods has been published by Snyder (1947). The principles of turbidimetry and their employment in bacteriological investigations is considered in the Appendix.

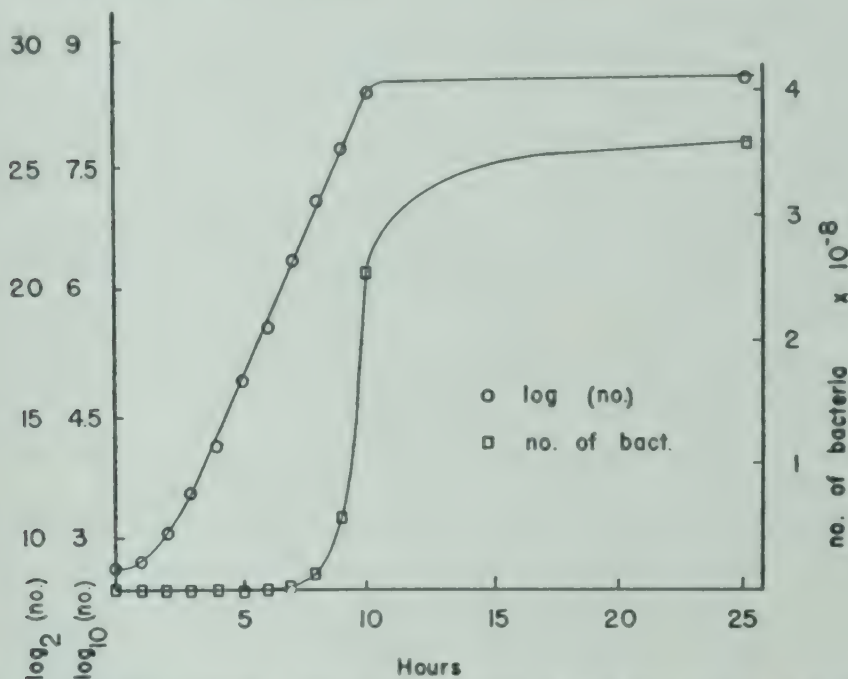


FIG. 31. Population growth curve of a culture of *Escherichia coli* plotted arithmetically and semi-logarithmically to both the base 10 and base 2 of numbers of organisms. (Multiplying log base 10 of a number by 3.322 will give the log base 2 of the number.)

Logarithm to the base 2 is the most useful of the plots since each unit on the ordinate represents a doubling in population. Thus by mere inspection or interpolation it is possible to determine the number of generations resulting in a given population and the generation time at any phase of growth. It is an extraordinary fact that the superior merit of the simple device of using logarithms to the base 2 in calculating and plotting growth curve data has been largely ignored though emphasized as recently as 1942 by Monod. Proof, indeed, that even in science the accepted ways are not always the best available.

#### THE BACTERIAL CULTURE CYCLE

Bacteria transferred into a new and favorable environment will grow and multiply with the result that the culture soon reaches a characteristic maximum population. This maximum population may be supported for some time but it eventually wanes to the point of extinction. A more intimate study and quantitative analysis of these events reveals a complex and characteristic cycle of phenomena to which the bacteriologist has devoted much attention.

By far the most of the investigations have been conducted with cultures developing in liquid media. There is no reason, however, to suspect any fundamental difference in the nature of the events occurring in liquid and on solid media except that characteristically a greater total population and density of organisms per unit volume of environment is attained on solid than in liquid media. Possibly this observation is explained in all cases by any one or a combination of three facts: 1) In the case of organisms utilizing free molecular oxygen as a hydrogen acceptor the availability of free oxygen does not become a limiting factor for growth on solid media so soon as it does with unaerated liquid media. 2) When the accumulation of toxic metabolic products is the limiting factor in growth, growth as an isolated colonial mass on a solid surface is advantageous since the concentration of metabolic products is reduced in the areas in which growth is concentrated by their free diffusion into the unoccupied areas of the solid medium. 3) When the availability of foods is the limiting factor for growth the unoccupied areas of solid medium provide a reservoir from which fresh foods are obtained by means of their free diffusion into the areas of colonial development.

The development of a bacterial culture may be conveniently studied by separating it into a series of connected and consecutive phases characterized by variations of growth rate. When plotted the quantitative data on changes in the amounts of bacterial protoplasm or numbers of organisms with time give a sigmoid or so-called bacterial growth curve (figure 31). The points of inflection on such a graph may be used to indicate the transition from one phase of development to another. While the phases of such a curve have not always been labeled identically by different authors it will be instructive to list the descriptive systems of nomenclature most popular at the present time:

<i>Buchanan (1917)</i>		<i>Monoð (1942)</i>	<i>Generation Time</i>
1. lag phase	} phase of adjustment (Winslow and Walker, 1939; Porter, 1946)	lag phase	very long
2. increased logarithmic growth phase		acceleration phase	decreasing
3. logarithmic growth phase		exponential phase	minimal and constant
4. decreased logarithmic growth phase		retardation phase	increasing
5. maximum or stationary phase		stationary phase	infinity or multiplication balanced by death rate
6. accelerating death phase		phase of decline	infinity or multiplication overbalanced by death rate
7. logarithmic death phase		phase of decline	



It will be noted that Buchanan's nomenclature uses the term logarithmic, an acknowledgement of the common practice of plotting logarithmic values, rather than arithmetic values of quantity.

The nature and sequence of the phases of the bacterial growth curve remain the same irrespective of whether the amount of bacterial protoplasm or the number of organisms are plotted. Nonetheless it has been found that the increase in bacterial protoplasm, or rate of growth, does not always coincide with the increase in population or with rate of multiplication. The greatest inconsistencies occur during the phase of adjustment. In plotting the bacterial growth curves it is therefore always necessary to make perfectly clear which quantity is being charted.

### *Phase of Adjustment*

On transfer of an inoculum to a fresh medium the organisms do not immediately begin to multiply at the maximum rate of which they are capable in the new medium. There may even be a temporary decrease in the number of organisms, but in any case this temporary lag or phase of non-existent or very slow development of the culture is eventually succeeded by a period of increasing growth rate, and finally by a phase of maximum and constant development before phases of decline set in. The lag and accelerating growth rate phases represent a phase of adjustment, a time during which the organisms are gradually adapted for growth and multiplication at a maximum rate under the particular conditions of environment.

The phase of adjustment has been visualized as a period during which the metabolism of the organisms is gradually building up to some steady state. But why is the steady state not possible from the very beginning of transfer into a new medium? What are the deficiencies in the environment or in the physiological state of the organisms which produce the observed phase of adjustment?

From 1895, when Müller first called attention to the lag phase, the literature has been rich in reports of studies attempting to answer these questions. The hypotheses proposed have been numerous and not always in agreement. Rather than review each of them a modern view will be presented which is not in contradiction to older ideas and which attempts to make use of the following well established observations.

- 1) While subject to many variables, the phase of adjustment is generally of greater duration for those species inherently possessing the longer generation times during the period of exponential growth.

- 2) Depending on the physiological age of the culture from which the inoculum is taken, the phase of adjustment varies greatly. In general the lag is greatest when the inoculum is from a culture in the stationary phase

and still greater when taken from the phases of decline in population. If the transfer is made into the same kind of medium from which the inoculum is taken there is no discernible lag providing the inoculum is from a culture already growing in the logarithmic phase. These observations permit the generalization that subcultured bacteria tend to multiply at the same

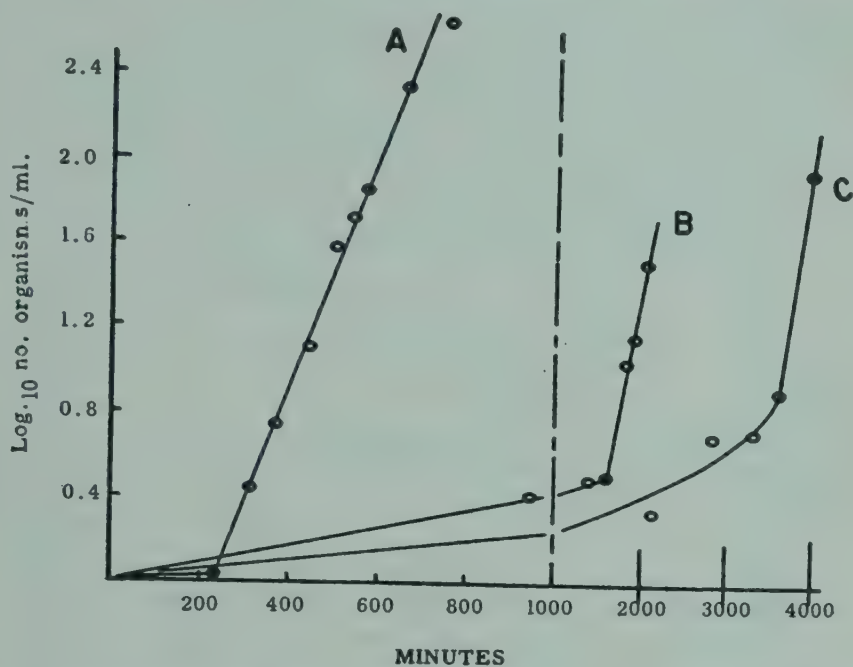


FIG. 32. Variations of the growth curve of *Escherichia coli* var. *mutabile* dependent on the nature of the medium from which the inoculum is taken when transferred to a given synthetic medium of ammonium sulfate, lactose, and salts.

Curve A. Ideal logarithmic form obtained when the inoculum is taken after a considerable number of subcultures in the synthetic medium.

Curves B and C. Irregular growth curves obtained in the synthetic medium when transfer is made without prior adaptation for growth in the same medium.

In the cases recorded, the source of the original inocula was a non-synthetic nutrient broth medium.

(Adapted from Hinshelwood, 1948)

rate at which they multiplied in the original culture. The rule is often violated when the transfer is made into a new kind of medium. In these cases the growth curves may be of irregular shape and show a lag phase irrespective of the cultural age of the inoculum (fig. 32).

3) The length of the lag phase decreases with an increased inoculum and quantitatively tends to be a linear function of the logarithm of the number of organisms in the inoculum.

4) Whatever the causes of the phase of adjustment they are without effect on the other phases of growth. This is illustrated by the growth

curves of Figure 32 which show practically the same slope during the period of exponential growth in spite of the very large differences in the extent of the lag.

5) In the phase of adjustment the rate of multiplication tends to lag behind the rate of growth except for diphtheroid bacilli. The result is a larger average size of organism than occurs during the other growth phases. Another consequence is a higher rate of metabolic activity such as oxygen consumption or carbon dioxide, ammonia, and heat evolution when measurements are expressed as activity per cell. The enzymatic activity of bacterial protoplasm per unit of dry weight or nitrogen is the same during the phases of adjustment and exponential growth (fig. 33) which lends

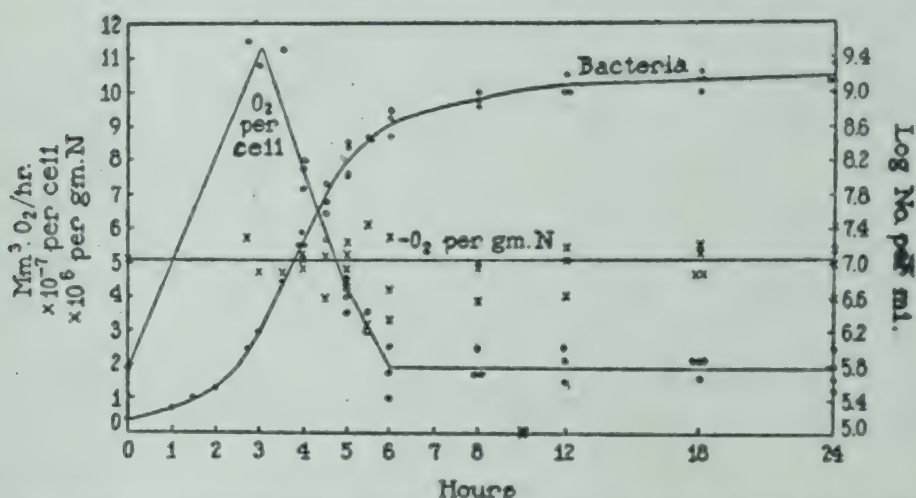


FIG. 33. Rates of oxygen uptake per viable cell and per gram of bacterial nitrogen, in relation to phase of growth of *Escherichia coli* cultures.

(From Hershey and Bronfenbrenner, 1938)

further credence to the view that the increased metabolic activity per cell in the phase of adjustment is only a question of the size of the individual organisms. Still another consequence of a lower rate for multiplication than growth is the finding that the length of the phase of adjustment as measured by estimation of bacterial protoplasm may be of lesser duration than when measured by population counts. As a matter of fact under ideal circumstances the phase of adjustment for growth may not appear while being quite evident as a phase when measured by multiplication.

6) The physical and chemical organization of bacteria is not fixed throughout the extent of the growth curve. Thus during the phase of adjustment and in the early period of exponential growth the organisms appear to be most permeable and most sensitive to sudden changes in environment. These organisms, often referred to as being in a state of "physiological youth", are easily killed by heat and cold and by transfer into solutions of



high salt concentration (Table 17). The electrophoretic mobility is then at a minimum. Suspensions at this age in normal serum, salt, and acid solutions show the greatest stability toward agglutination. The percentage of total water in the organisms is also greatest in the stage of "physiological youth". Possibly this may be in part responsible for the greater sensitivity of these cells to such a physical agency as heat.

The basophilic staining properties of the organisms are also most intense at the early stages of growth. This property no doubt reflects the experimentally established fact of a proportionally greater concentration of nucleotides<sup>4</sup> in organisms from a young culture (fig. 34). The increase in the nucleotide content of the bacilli during the phase of adjustment is chiefly an increase in pentose type nucleic acid (fig. 35), while the desoxypentose nucleic acid content fluctuates only slightly and is almost independent of

TABLE 17

*Influence of age of culture upon survival of Escherichia coli when transferred to 5 per cent NaCl solution*

AGE	ORGANISMS	MORTALITY AT 1 HOUR IN 5% NaCl
<i>hrs</i>	<i>ml</i>	%
0	96,000	14
1	80,500	25
1.5	90,500	55
2	143,000	77
2.5	255,000	94

(From Sherman and Albus, 1924.)

the growth phase. A maximum pentosenucleic acid content of the individual cells occurs in the transition from lag to the exponential phase of growth and is gradually reduced during the exponential phase. Of great significance are the relationships found between the concentration of inoculum and both the nucleotide content and volume of the individual organism (fig. 36). An increased inoculum reduces the nucleic acids, volume, and the length of the lag phase. This correlation is a key observation in support of the modern explanation of the phase of adjustment which will now be discussed.

During growth in the isolated bacterial culture there is a simultaneous decrease in available consumable nutrients and an accumulation of metabolic products and the products of the decay of dead and dying organisms many of which are toxic. As a result the synthetic capacity of surviving

<sup>4</sup> The term nucleotide as used in this section signifies materials with the absorbancy characteristics of purine and pyrimidine groups when the organisms are examined spectrophotometrically.

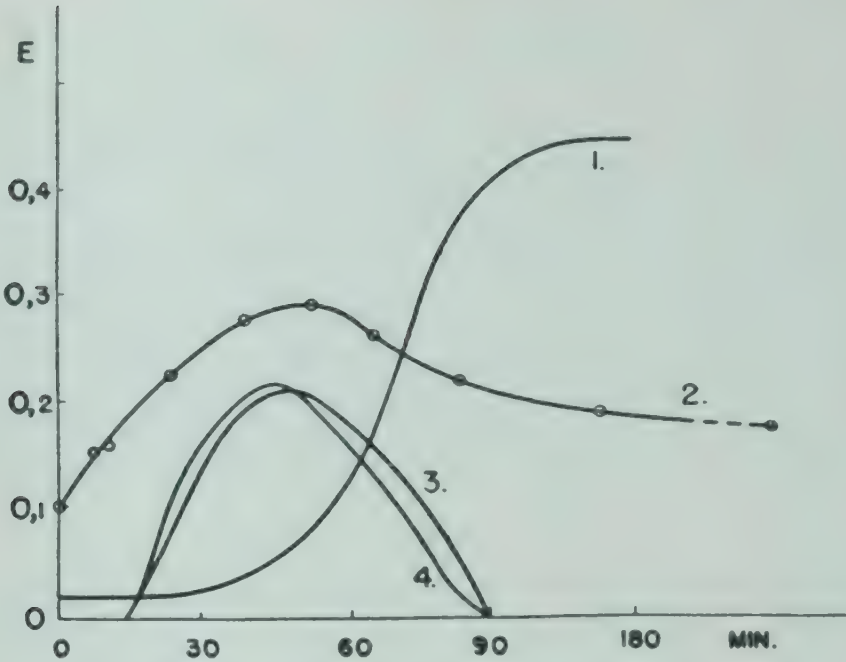


FIG. 34. Early phases of the growth curve of *Bacillus cereus*

1. Bacterial population curve
2. Nucleotide content of individual bacilli estimated from absorbancy data obtained at 258  $m\mu$
3. Rate of multiplication curve
4. Increase in nucleotide content of individual bacilli

(From Malmgren & Hedén, 1947)

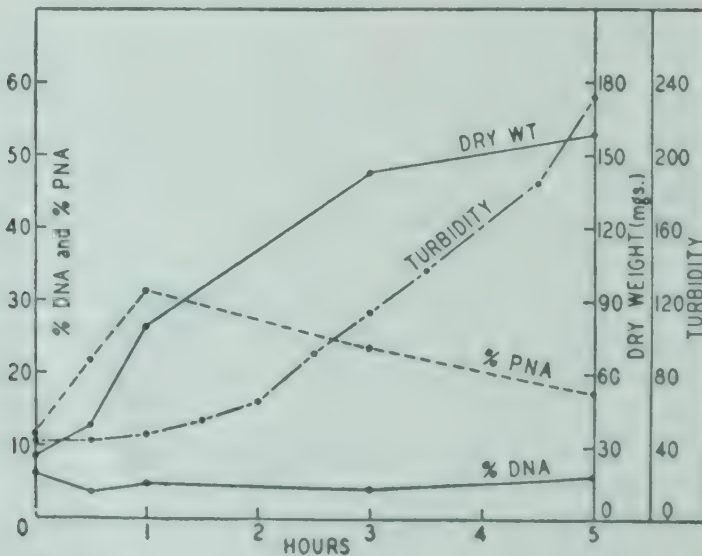


Fig. 35. Dry weight, turbidity, and the desoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) content of a culture of *Proteus vulgaris* in the early phases of growth.

(From Levy, Skutch, and Schade, 1949)

cells is gradually impaired and the rate of growth decreases. The continued growth of a culture at a constant and maximum rate as represented by the exponential phase is only possible so long as a steady state can be maintained. Continued depletion of nutrients and accumulation of toxic products make this steady state impossible since they will adversely affect the

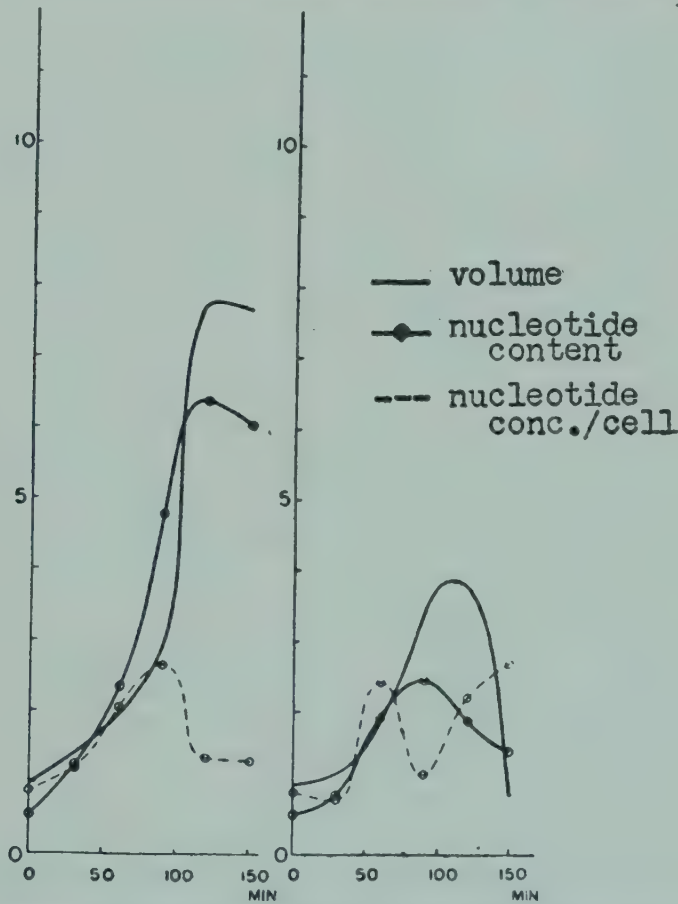


FIG. 36. Illustration of the dependence of nucleotide content and volume of *Proteus vulgaris* on the amount of inoculum. Inoculum was eight times larger in the experiment recorded on the right.

(From Malmgren and Hedén, 1947)

capacity of the protoplasm to maintain the relative concentration gradients of reactants (intermediate metabolites) necessary for the continued existence of the steady state. The result is a reduction in the overall synthetic capacity and a change in cell chemistry and physical organization.

It is probably significant for this view that as one aspect of reduced synthetic capacity the organisms from old cultures do not readily synthesize adaptive enzymes. This synthesis is accomplished most readily by organisms just beyond the lag phase.

When organisms from cultures beyond the exponential growth phase



are transferred to a fresh medium where nutrients are in good supply and toxic products of metabolism absent, growth will not begin at a maximum and constant rate until the cells have passed through a phase of adjustment. The phase of adjustment will involve the replenishing of the internal supply of intermediate metabolites and a gradual building up to a steady state.

Growth is in part the synthesis of protein. Evidence increasingly points to the active participation of pentosenucleic acid in this synthesis. Therefore the buildup to a steady state in the production of protein requires a preceding accumulation of the necessary quantities of pentosenucleic acids as well as intermediate metabolites. The phase of adjustment, then, is the period in which these needs are satisfied, but since many of the intermediate metabolites may be diffusible through the cell boundaries the quantity of inoculum as well as its age would be expected to influence the extent of the phase of adjustment. The larger the inoculum the greater the amount of such essential intermediate products transferred both as a part of the organisms themselves and in solution in the culture broth, and therefore the shorter the lag period which represents the time necessary for synthesis of adequate quantities of such products. In Figure 37 an experiment is illustrated indicating the synthesis during the lag of the postulated intermediate products to necessary levels and the relationship to the nucleotide content of bacteria.

The complex and nonsynthetic media in which bacteria are so often grown in the laboratory may contain toxic materials. Evidence is accumulating that these may include heavy metals and fatty acids or other compounds of a lipoidal nature. These poisons may cause a delay in growth varying with their concentrations. The use of a large inoculum may reduce the relative effective concentration of these poisons by adsorption to colloid in the transferred medium and the surfaces of the organisms and thus reduce the lag period. In this connection the phenomenon of dormancy must be mentioned. *Dormancy* has been defined as a prolonged quiescence of a few cells of a transplant after the majority have multiplied and is to be distinguished from the normal lag we have just considered which is a latent period of only a few hours' duration. In one case dormancy of 72 months' duration has been reported for the endospores of *Clostridium botulinum*. Dormancy is not inherent in the nature of a bacterium but rather is a function of the toxicity of the medium. Pretreatment of media with adsorbents such as activated carbon effectively reduces the dormancy as compared to organisms observed in untreated control media.

### *The Exponential Growth Phase*

A period of steady and rapid development of a bacterial culture or the exponential phase of growth succeeds the phase of adjustment (see fig. 31).

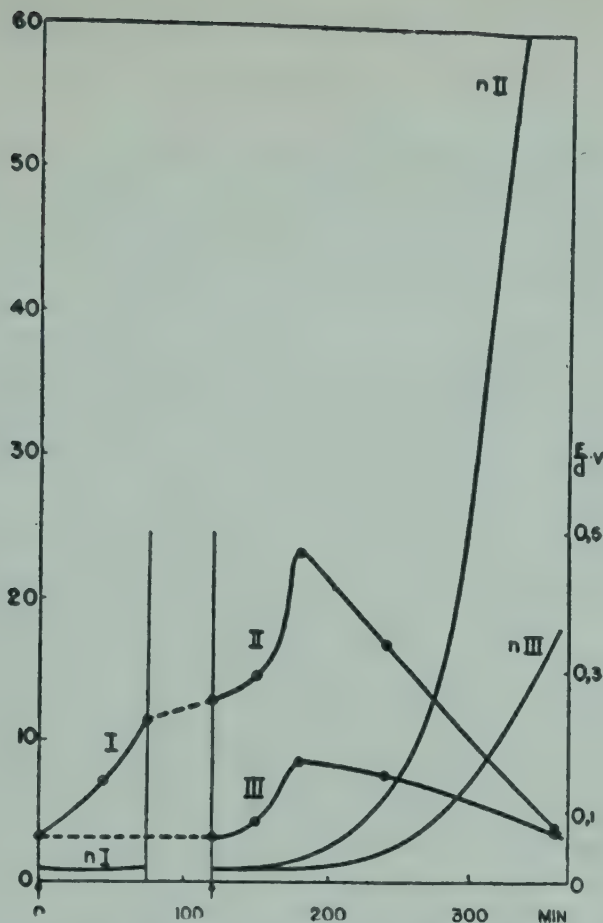


FIG. 37. Experiment with *Proteus vulgaris* designed to test the hypothesis that during lag phase the synthesis of a diffusable essential factor occurs which is associated with nucleotide metabolism.

(From Malmgren and Hedén, 1947)

An 18 hour culture (I) was inoculated into fresh broth. At the end of 75 minutes, at which time the maximum nucleotide content of the bacteria was expected, the culture was centrifuged. The cells (II) were resuspended in fresh broth. An inoculum of a fresh 18 hour old culture (III) was added to the supernate from the centrifuged culture. It is readily apparent that the inoculation of fresh cells into medium previously inoculated results in a decrease in the normal nucleotide content of the bacilli and in a normal lag phase, whereas the cells transferred 75 minutes after preliminary inoculation and growth into a fresh medium had an unusually high nucleotide content and a shortened lag phase. Population curves are marked n.

It may be argued that the organisms in series III are referred to the wrong time scale and that the proper point of reference would be the time of inoculation into the medium previously used for growth. Viewed in this light it may be questioned whether the lag period is any longer for III than II.

A point of potential interest in the formation of nucleotides in II occurs after the reinoculation. It appears from the figure that a new lag period arises since the curve does not ascend sharply for some minutes when contrasted with the steep slope observed before centrifugation. This lag in nucleotide synthesis may conceivably be due to a lack of a necessary material synthesized by the cells such as  $\text{CO}_2$ .  $\text{CO}_2$  concentration might be higher after a period of growth than in a fresh medium. The student should realize that an important control in this type of experiment would be the reintroduction of bacilli of the type of II into the same medium from which they came. This procedure would reveal any influence of centrifugation, and handling of the organisms, as well as alterations of concentrations of gases in the medium.



During exponential growth the rates of accretion of protoplasm and of multiplication bear a constant relation to one another so that a measure of one may safely be taken as an index of the other. We of course have warned against this procedure when one is concerned with the other cultural growth phases.

The rate of multiplication during exponential growth is an inherent property of the species, organisms such as the gram negative intestinal bacilli being capable of doubling in number at 15-30 minute intervals while the tubercle bacillus of man even under the best known conditions rarely divides in less than two or three hours of growth.

For any given set of conditions and species an optimal temperature exists for most rapid growth. Below and above this temperature the rates will be reduced, falling slowly at the lower temperatures and precipitously at the higher temperatures (fig. 29). The duration of the exponential phase decreases with a rise in temperature up to the optimal temperature. The effect, however, is such that the ratio of the generation time to the duration of the exponential phase tends to be constant. Such a result indicates that factors which limit the duration of exponential growth come into play sooner at the higher temperatures but do not affect the overall efficiency of protoplasmic synthesis when measured as the total amount of product accumulated during the exponential phase at all temperatures up to the optimum temperature.

The chemical nature of the media (and, with aerobes, the extent of aeration) will affect the growth rate. Transfer into a medium of different composition from the mother culture may result in a lower growth rate, but the rate can usually be increased by adaptation by means of continued subculture of the organism in the new medium. However, no amount of subculturing of a given organism will result in the same growth rate in all media. Some media remain definitely inferior to others. This is particularly true for many heterotrophic bacteria cultured in synthetic media.

Variations in the pH of media seem to have less effect on the growth rate than on the total yield of growth. Furthermore, the growth rate is dependent on variations in food concentration only in dilute media (fig. 38). The effect of  $\text{CO}_2$  on growth rate extends likewise over only a relatively low range of concentration of this substance (fig. 39).

The exponential growth phase comes to an end when food concentrations have dropped either to zero or to limiting values or when toxic metabolic products accumulate in sufficient quantity to upset the steady state. There seems to be no inherent reason why this growth phase should not go on indefinitely if the food supply could be maintained and the toxic products of metabolism eliminated. To this end there has been expended a considerable amount of successful effort in designing specialized apparatus.



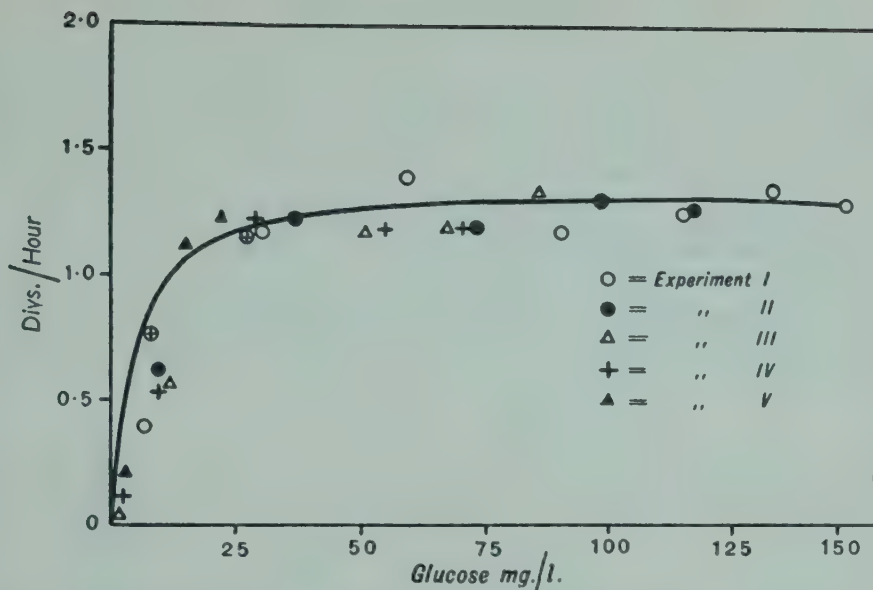


FIG. 38. Relationship of the multiplication rate to concentration of food.  
(From Monod, 1942)

Note that while the author has idealized the curve by having it originate at the origin, the data are such that it would be permissible to have the curve originate at some positive value of the abscissa. The choice is important since as plotted the curve assumes there is no small concentration of glucose below which growth is impossible. It would be important to know whether or not the assumption is valid.

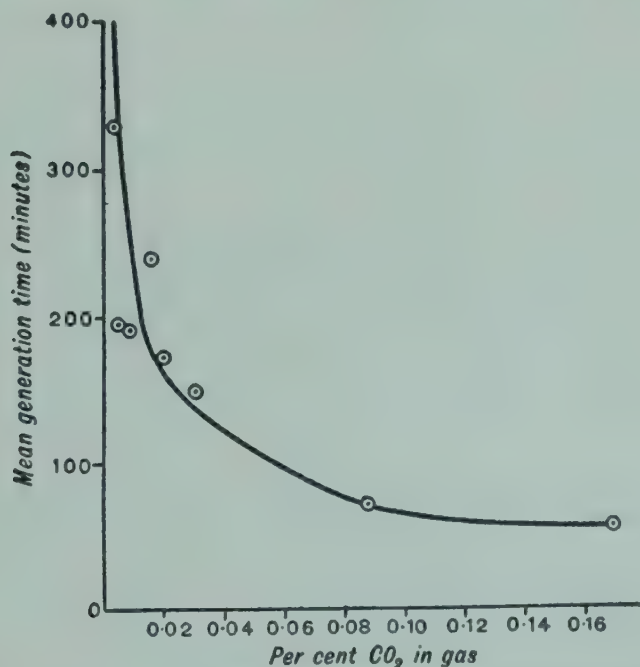


FIG. 39. Relation of the multiplication rate to the concentration of CO<sub>2</sub>.  
(From Hinshelwood, 1948)

The curve seems to approach the  $y$  axis asymptotically. The theoretical inference from this observation would be that there is some small limiting concentration of CO<sub>2</sub> below which multiplication does not take place.

A simple laboratory means of extending the period of active growth of bacterial cultures is to add activated carbon or some such adsorbent to a culture for the adsorption of toxic metabolites as they accumulate during growth. Still another successful scheme has been to grow bacteria within dialysis bags surrounded by large quantities of sterile medium. The virtue of this method lies in the opportunity for replenishing consumed food from the reserve in the external fluid while at the same time reducing in concentration the toxic metabolites within the bag by their outward diffusion.

### *The Decreasing Growth Phase*

The decreasing growth phase is expected to occur irrespective of whether the limitation of growth is due to exhaustion of food or to the accumulation of toxic metabolites. Since growth in an environment of limited extent is accompanied by a decreasing concentration of food the growth rate will fall when the food concentration has been reduced to appropriate levels (fig. 38). On the other hand, in the presence of excess food the accumulation of toxic metabolites must eventually reach concentrations high enough to make their presence felt. Since the accumulation of these toxic products is gradual there is no radical break in the growth curve at the end of exponential growth but rather a continuous deceleration of growth between this phase and the next or stationary phase.

### *Arithmetic Linear Growth*

Rarely, plots of the arithmetic number of bacteria in a developing culture against time will yield a straight line. Such a finding of arithmetic linear growth reflects an increase in generation time at a fixed rate. The slowing up of growth must signify the effective influence of some limiting factor. Unlike the decreasing growth phase where the deceleration in multiplication is increasing with time because of the expanding influence of a limiting factor for growth the cause of an arithmetic linear growth curve must be constant with time. For this reason exhaustion of food supplies and accumulation of toxic metabolites, factors which become more pronounced with time, cannot be the limiting factors responsible for arithmetic linear growth. The concentration of some material whose solubility is fixed and limiting such as oxygen or carbon dioxide might be involved in the phenomenon. In any case in the actual examples recorded in the scientific literature the exact cause has not been traced.

### *The Stationary Phase*

The exhaustion of nutrients and the accumulation of toxic materials together or singly must eventually cause a halt in the growth of a culture.

Bacterial cultures are notoriously difficult to buffer and to poise so as to maintain a constant pH and oxidation-reduction potential. Thus changes of these variables to levels not conducive to continued growth account frequently for the passing of the exponential phase of growth.

If the population of a bacterial culture is plotted against the concentration of food a linear relationship is obtained up to some maximum value.

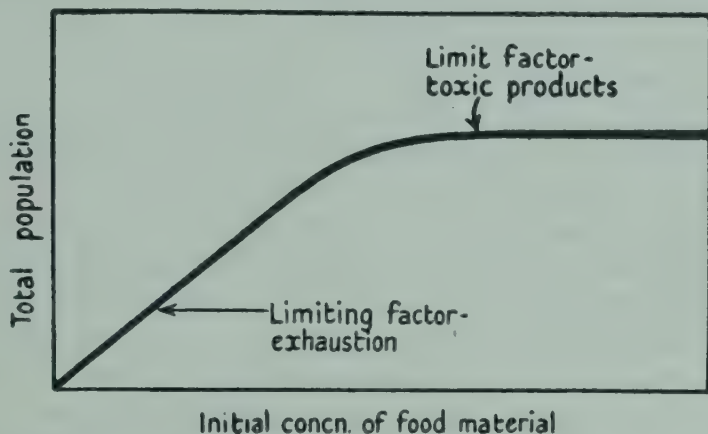


FIG. 40. Relation of total population to concentration of food.

(From Hinshelwood, 1946)

The equation of the straight line where food exhaustion is the cause of cessation of growth is  $y = ax + b$ , where  $a$  is the slope and  $b$  the intercept on the  $y$  axis (Monod, 1942, used the symbol  $K$  to represent  $a$ ). Since the intercept in the above case is at the origin, the equation reduces to  $y = ax$ . Since  $y$  is the total growth obtained with  $x$  concentration of food, the slope of the curve expresses quantitatively the relation of the quantity of food to the amount of growth or synthesis sustained by the food. To repeat, this relationship can be obtained only by working with bacterial cultures under circumstances where the concentration of the nutrient is the only known limiting factor for growth. Therefore, while it is theoretically possible to determine the amount of synthesis supported by a unit quantity of nutrient from data obtained by growth at only one limiting concentration of food, in actual practice total growth must be studied for several concentrations. In other words, one is under the necessity of empirically establishing the range over which the concentration of food is the only limiting factor for growth.

Beyond this concentration the relationship no longer is maintained, the influence of harmful metabolic products becoming the limiting factor for growth rather than food concentration. The relationship is illustrated in Figure 40. It will be noted that the straight line portion of the curve is drawn to intercept the origin. This treatment implies that growth can take place in exceedingly dilute medium. There is some evidence both for and against this assumption. Actually at the present time the data are so few as to require that individual investigators satisfy themselves as to the existence of a limiting or threshold concentration of food below which



growth does not occur, for the particular conditions and species in which they are interested.

The slope of the portion of the curve showing a linear relationship of total growth to nutrient concentration (fig. 40) is a measure of the growth possible per unit of food consumed. It has been found to be a remarkably reproducible value. Thus it provides a ready means for the quantitative comparison of the value of nutrients of a given medium under different conditions or of the capacity of different organisms to assimilate a given nutrient. For example, with *Escherichia coli* the determination of the slopes obtained on plotting total population against various limiting quantities of different sugars as carbon source has shown that the total growth is independent of the nature of the carbohydrate.

Temperature is a most important variable in determining total growth, and the greatest crops are obtained below the temperatures giving the most rapid growth. There are few data attempting to relate the influence of the temperature on total growth to the nature of medium constituents. In the case of *Escherichia coli* in a synthetic medium the effect of temperature was found to be independent of the nature of the carbon source.

### *M Concentration*

With any fluid culture the total yield of bacteria per unit volume of medium tends to be constant for a given species. This population of bacteria has been labeled the *M concentration* by Bail. Frequently one may remove the organisms at an *M* concentration by centrifugation and note that a fresh inoculum will result in growth. In these cases the cessation of growth by the original culture cannot have been due to either the exhaustion of food, or accumulation of toxic metabolic products. Observations of this character have suggested that a certain amount of physical space which has been thought of as "biological space" is required to support the growth of individual bacteria. This, however, is a rather indefinite view that does not lead to any increase in understanding of the nature of the phenomenon being described. An unexplored and plausible hypothesis is that for growth and multiplication to occur a critical minimum concentration of food per unit of surface or volume of organism must be exceeded. This of course is suggested by findings that in very dilute media in spite of the presence of some food no growth and multiplication may occur. In such cases one might expect from considerations of the steady state that in very dilute media the concentration gradient and the quantity of metabolite diffusing would be quite small. Under such circumstances a minimum quantity of metabolite might permit a low metabolic rate in the cell but one insufficient for increase in bulk to the critical volume necessary for multiplication.

With the ordinary methods of cultivation the population within a culture

increases with an accompanying decrease of food concentrations. Thus the available food concentration *per organism* is declining continuously and multiplication may cease when a critical level is reached. On the other hand, when the organisms are removed, and a few inoculum organisms added, the concentration of food per organism has been increased relatively and growth can be reinitiated. It is significant for this point of view that the second growth does not result in as large a population as the first growth.

### *Phase of Decline*

The stationary phase is followed by a stage of decreasing population. Although there has been much practical interest in problems of sterilization and chemical disinfection and no lack of studies on the killing of bacteria by chemical and physical agents, there are surprisingly few reports on the normal causes of death of bacteria in ordinary nutrient media. In considering the nature of the phase of decline it is necessary to recognize this lack of information and to proceed into a discussion of the subject on the basis of intelligent guesses.

The cause of death after the period of active growth of a culture may reasonably be thought to be related to the nature and concentration of the limiting factor responsible for the cessation of growth. When the exhaustion of food is responsible for the end of development the organisms, though incapable of further growth, are not immediately deprived of the means for catabolic activity. Any internal food reserves, intermediate metabolites, and finally the intimate structure of the organisms themselves become the fuel for the continued respiratory activity of the organism. It may be readily visualized that this consumption of structural substance cannot go on indefinitely without at some point permanently destroying the capacity for growth even when the exhausted organisms are transferred to a fresh medium. The number of viable organisms counted in the phase of decline though decreasing with time, will vary in absolute number depending on the quality of the medium used in subculturing and enumeration of the organisms.

When the growth of a bacterial culture is brought to a halt by the accumulation of toxic metabolic products the cause of death must depend on the nature of the poison. The most dramatic cases, such as those of pneumococcus and meningococcus cultures, are often observed to become sterile in amazingly short periods of time. It is not uncommon to lose specimens of these species recently isolated from natural sources if they are not religiously transferred at short intervals of 72 hours or less.

Unfortunately there is only a minimum of information on the nature of surmised toxic metabolites and their mode of action. There would seem to be open here a large field for future exploration and the possibility for



the discovery of specific *auto-antibiotics*, antibiotics effective against the very organisms producing them. In this connection the observation should be recalled that with some species of bacteria plates of solid media crowded with colonies do not show any tendency for the colonies to overgrow and run into one another.

The presence in a medium of a sufficient quantity of a fermentable carbohydrate will result in an accumulation of organic acids as end products of

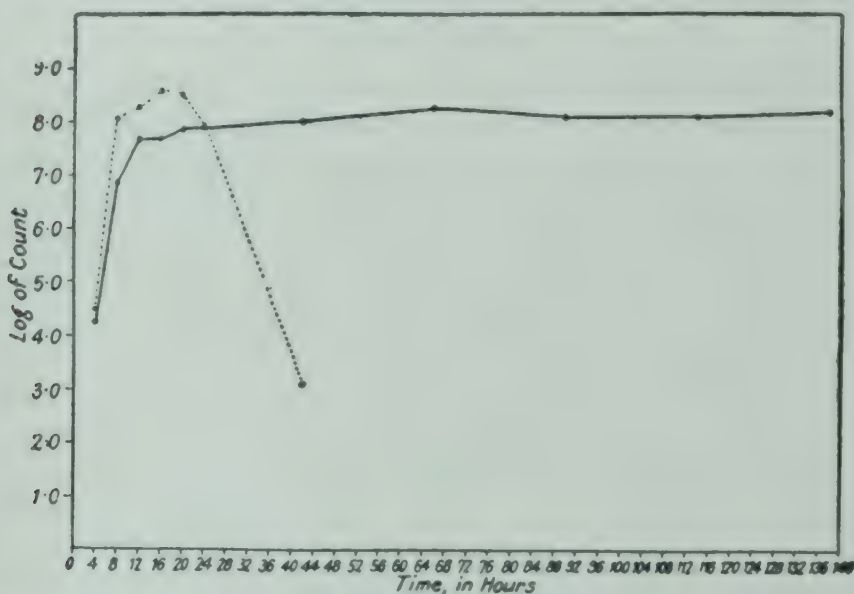


FIG. 41. Relation of the phase of decline to the nature of the medium in the case of *Salmonella typhimurium*. (—sugar absent; . . . sugar present)

(From Topley and Wilson, 1946)

Note that in the medium with a fermentable sugar the growth curve shows only a short stationary phase in contrast to the medium free of sugar. The fermentation of sugar results in the rapid accumulation of toxic concentrations of organic acids which are responsible for the events pictured. Note too that the decrease in the viable population after the stationary phase is exponential, a circumstance which can be duplicated by exposing sensitive bacteria to acidic environments.

metabolism and in a drop in pH values to harmful acidic levels. Under these conditions the phase of decline has the characteristics associated with the killing of bacteria by harmful concentrations of organic acids. Such a decline in population may be exponential as shown in Figure 41.

Without fermentable sugars in the medium the phase of decline is rarely exponential. It is often so irregular in character as not to be easily fitted to some obvious mathematical equation. A case of this type is recorded in Figure 42 which illustrates another interesting aspect. It shows that when the total population of a culture is measured by a method which does not distinguish dead from living organisms, an apparent stationary



phase of growth or a phase of very slow addition to population is maintained for a much greater length of time than would be suspected from data on viable organisms alone. In these cases the stationary phase cannot be looked upon as a period during which absolutely no growth and multiplication are going on but rather as a phase during which reproduction is going on in sufficient measure to compensate for the death of organisms in spite of a significant death rate. It would not be expected that such a situation would arise in a case where the concentration of food has been the limiting

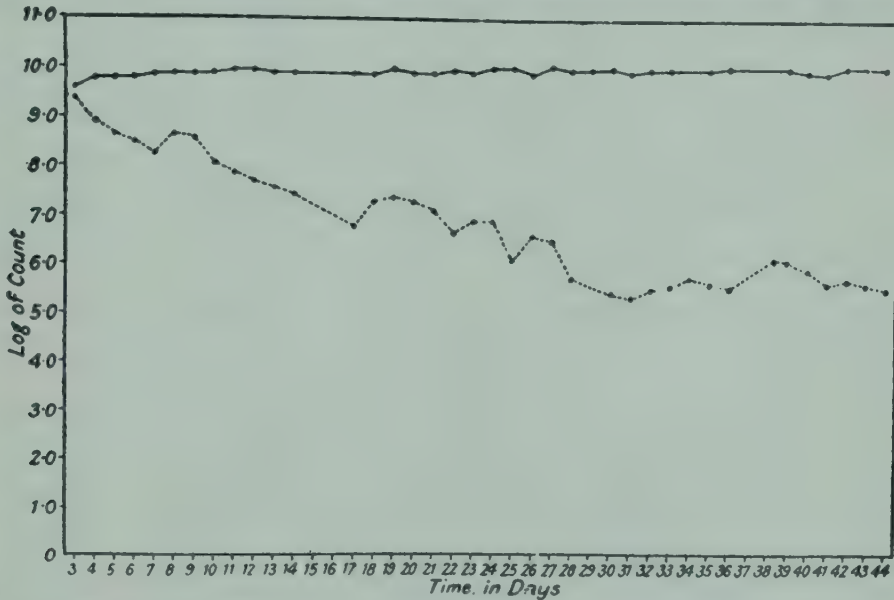


FIG. 42. Phase of decline of a culture of *Salmonella typhimurium*.

(From Topley and Wilson, 1946)

In the viable count curve, note the occurrence of multiplication in spite of a continuous decline in the number of living organisms. The irregularities of the curve for viable count are considered significant, and the rises may mark the points at which selection operates to favor the growth of different mutants.

factor of growth, for once the available food has been used growth and reproduction must cease. Therefore, this sort of situation may be expected to occur only in media with excess food where accumulation of metabolic end products to harmful levels is possible.

The finding of a high death rate in some cultures during a phase of reduced multiplication raises the question as to whether or not death of bacteria may not be occurring throughout the growth cycle of the culture. Some data are presented in Table 18 which show that death of organisms may occur even during the period of most rapid growth. It has been suggested, and there is some experimental support for the idea, that the very best media will not show any significant proportion of dying bacteria during

the active growth phases. This situation is particularly probable when the culture is a clone. Since all the descendants of a single, asexually reproducing organism would have the same hereditary constitution there should be few, if any, deaths in media with a full complement of required nutrients, free of added poisons, and where food concentration alone limits growth. In such media with an excess of food the growth phases prior to the accumulation of toxic materials responsible for the cessation of exponential growth should not show any significant death rate. In dealing with a clone in an optimal environment lethal mutations are the only obvious causes of

TABLE 18

*Evidence of a significant death rate for Salmonella pullorum during exponential growth in a nonsynthetic nutrient broth*

TIME	TOTAL COUNT PER CUBIC CENTIMETER	VIABLE COUNT PER CUBIC CENTIMETER	NUMBER OF DEAD ORGANISMS	PERCENTAGE OF VIABLE TO TOTAL
<i>min</i>				%
0	1,303,000	1,198,000	105,000	91.89
50		1,699,000		
110		2,983,000		
170		12,480,000		
220	53,440,000	35,690,000	17,750,000	66.77
270	156,000,000	111,000,000	45,000,000	71.15
320	345,500,000	238,700,000	107,000,000	69.08
370	563,700,000	339,900,000	223,800,000	60.30
420	731,300,000	451,200,000	280,100,000	61.70
470	840,300,000	553,400,000	286,900,000	65.86

(From Wilson, 1922.)

death, but rates of mutation are so low as to be precluded as a cause of death when death rates are as high as those which have been observed.

### MATHEMATICS OF THE BACTERIAL GROWTH CURVE

It is often desirable to describe the phenomena of the growth curve in mathematical terms. The advantage deriving from the application of mathematical reasoning to problems of the growth curve are those associated with the usual mathematical expression of scalar and vector phenomena. It permits a preciseness of definition and an economy of expression in quantitative terms otherwise unattainable. Not infrequently hidden relations may be revealed. Most important from the point of view of the investigator who is interested in comparing the effects of given variables on particular growth phenomena, a mathematical treatment permits quantitative evaluation of effects which otherwise might be revealed in only an indefinite qualitative way. In what follows no attempt has been made to

treat the mathematics of the growth curve exhaustively. Rather there have been outlined the basic minima of useful procedures with which all bacteriologists should be acquainted.

#### THE PHASE OF ADJUSTMENT

In order to compare the influence of variables in the phase of adjustment it is desirable to be able to compute a value for this period of reduced

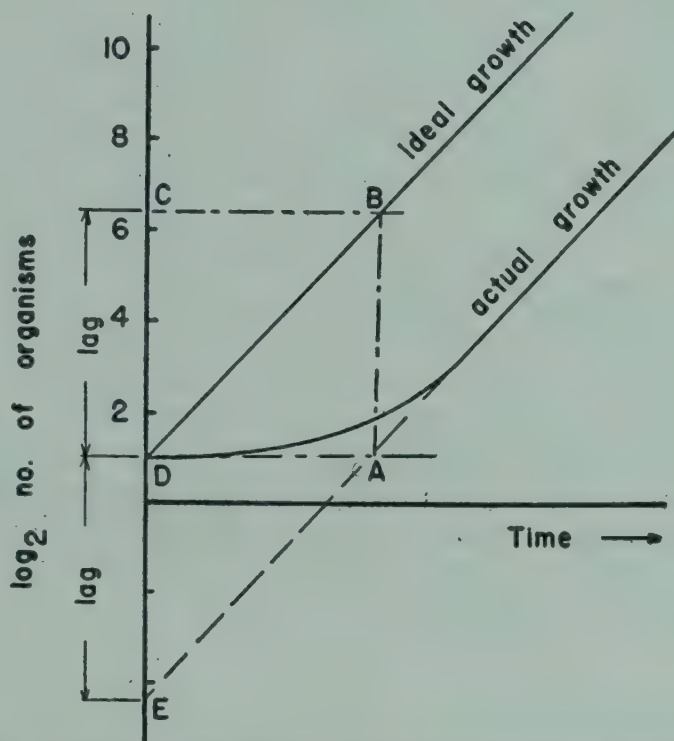


FIG. 43. Two geometric methods for the determination of the growth lag.

By plotting populations to the log base 2 it is possible to estimate by inspection that in the case illustrated 5.5 generations did not appear in the multiplying culture.

growth rate preceding the exponential phase of growth. A suitable and easily determined value is the amount of growth or number of divisions or generations lost to the development of the culture by this period because growth is at a slower rate than that attained during exponential growth. This value has been called the *growth lag* ( $L$ ) by Monod. It can be obtained by geometric means. In one method the steps are as follows: 1) The logarithmic growth curve is plotted preferably employing the base 2 logarithms. 2) A straight line is drawn from the point of origin of the growth curve parallel to the abscissa (time coordinate or  $X$  axis). 3) Then the exponential portion of the growth curve is extrapolated to cross the line previously drawn. The distance from this point (point A, fig. 43) to the



ordinate represents the difference between the time the culture developed to any given quantity of growth or population and the time in which this same quantity of growth or population would have been achieved if development had been exponential from the time of inoculation into the medium. 4) This distance is used to locate the "ideal growth curve" for the culture, one lacking the phase of adjustment and entirely exponential in character, by drawing a line from the point of origin parallel to the observed growth curve. 5) A perpendicular at point A is then drawn to the "ideal growth curve" and from the point of intersection (B) on the "ideal growth curve". 6) Another perpendicular is drawn to the ordinate or  $y$  axis. The distance from the origin of the growth curve to this point of intersection on the ordinate (point C, fig. 43) represents the growth lag in terms of growth not achieved because of the phase of adjustment.

#### THE EXPONENTIAL GROWTH PHASE

Since bacteria multiply by binary fission each doubling of the number of organisms in a culture represents one generation. Therefore, if the number of organisms at the beginning and end of a time period are known the number of generations or divisions can be calculated by means of a simple geometric progression. Obviously, the number of organisms  $y$  at the end of a time period equals the number  $x$  at the beginning multiplied by 2 (since each organism divides into two) raised to the power  $n$  representing the number of generations occurring in the time interval. This relationship, of course, can be expressed as an equation.

$$y = x2^n \quad (1)$$

taking  $\log_{10}$  of both sides

$$\log_{10} y = \log_{10} x + n \log_{10} 2 \quad (2)$$

and

$$n = \frac{\log_{10} y/x}{\log_{10} 2} \quad (3)$$

The scheme is still simpler if logarithms to base 2 are taken. In this case it will be recalled that  $\log_2 2 = 1$  and equation (2) then becomes

$$\log_2 y = \log_2 x + n \quad (4)$$

and

$$n = \log_2 y/x = \log_2 y - \log_2 x \quad (5)$$

This equation is related to (3) by

$$n = \log_2 y/x = \frac{\log_{10} y/x}{\log_{10} 2} = \frac{\log_{10} y/x}{0.301} \quad (6)$$

If the length of time it takes for a doubling of population or the *generation time* ( $g$ ) is wanted, the number of generations occurring in the time interval studied need merely be divided into the time  $t$ :

$$g = \frac{t}{n} \quad (7)$$

When there is any significant rate of death during the exponential growth phase the methods of calculation outlined are inaccurate and err on the side of a conservative estimate of the actual generations and generation times. This result is true because the calculations assume that all of the organisms multiply. If there is a significant death rate during the growth period the total increase in population is actually due to multiplication of fewer organisms than assumed, and it is necessary to take this into account by decreasing the denominator of equation (3). This correction may be made by substituting for  $\log 2$  the logarithm of twice the per cent of viable to total cells at the end of the time period of population increase. The per cent viable cells in the population is obtained by doing both total and viable counts on the culture.

The *growth rate*,  $R$ , during exponential growth is represented by the slope of the straight line in a plot of the logarithmic growth curve. The slope (and thereby the growth rate) is simply calculated by employing the traditional equation which is in terms of the variables of the growth curve:

$$R = \frac{\log_2 y_2 - \log_2 y_1}{t_2 - t_1} = \frac{\log_2 y_2/y_1}{t_2 - t_1} \quad (9)$$

It should be recognized that the above methods of computation result in expressions of average values of the events occurring during the interval of time covered by the data used in substituting in the formula. This state of affairs is perfectly satisfactory when the growth phenomena are occurring at a constant rate as is the situation in the period of exponential growth. The equations are not a true representation of actual facts for periods of growth when the rates are fluctuating. Thus, at every instant during the accelerating phase of growth the growth rate is changing, and the calculations outlined above cannot be applied, and calculus must be used to solve the problem. The mathematical statement of the problem and the derivations of equations need not be outlined here since they are generally available in numerous texts.<sup>5</sup> The formula most often seen in the bacteriological literature yields the constant  $k$ , the value for the rate of increase per cell during the period of growth also known as the growth rate constant

<sup>5</sup> A most excellent recent review is included in chapter 16 of the book by Brody (1945).

of Slator (1916) or the velocity coefficient of growth,

$$k = \frac{2.3}{t} \log_{10} \frac{b}{B} \quad (10)$$

where  $t$  is the time,  $B$  the number of bacteria at  $t = 0$ , and  $b$  the number of bacteria at the end of the given time.

#### THE STATUS OF EQUATIONS FOR THE GROWTH CURVE AS A WHOLE

Ever since the forceful presentation of the problem of population growth made by Malthus in 1798, biologists have been greatly interested in developing theories of growth and its mathematical expression. From numerous theories including those proposing a resemblance of the growth phenomenon to autocatalytic or allelocatalytic chemical reactions, the various equations of the growth curve have been derived. With these equations it is possible to predict growth curves and to compare them with actual curves. Curiously, equations based on a number of different theories do successfully predict actual curves. However, an equation which gives a mere coincidence of numbers is of no theoretical use to the biologist unless it depicts or suggests the *modus operandi* of the biological phenomenon. Equations based on different theoretical assumptions do about equally well in predicting actual bacterial growth curves which, unfortunately, are necessarily based on data obtained by methods not accurate enough to distinguish the relative value of closely approximating equations. Therefore, we must conclude that the present use of such equations of the growth curve as a whole have little practical value for increasing the insight of bacteriologists into the problems of bacterial growth.

The growth curves of a bacterium, of a bacterial culture, and of numerous phenomena of physics, chemistry, biology, and human society are sigmoid in nature. One may say there exists almost an infinity of processes having a sigmoid character. Thus the mere fact that a bacterial growth curve is of sigmoid shape cannot prove that growth is analogous to another phenomenon with the same type of curve of development although this false assumption has been made on several occasions. The situation has been summed up neatly by Thompson (1944) who states, "When the same curve depicts the growth of an individual, and of a population, and the velocity of a chemical reaction, it is enough to show that the analogy between these is a mathematical and not a physico-chemical one. The sigmoid curve of growth, common to them all, is sufficiently explained as an interference effect, due to opposing forces such as we may use a differential equation to express: a phase of acceleration is followed by a phase of retardation, and the causes of both are in each case complex, uncertain or unknown."



Now to Malthus must also be credited the original keen observation that the growth of biological populations involves interference effects or limiting factors. As he stated it, "There is no bound to prolific nature in plants and animals but what is made by means of their crowding and interfering with each other's means of subsistence." For bacteria this observation suggests an experimentally verifiable fact, namely, that the chronological age of a culture is of little biological importance. The rate of growth is a function of the density of bacterial population, not the age of the culture. Only under the most rigidly controlled and reproducible conditions can the parameter of time accurately reflect the population density and the related physiological state of the culture.

It has also been firmly established that growth lag, rate of exponential growth, and total growth or population are definite and easily calculated quantities which vary independently of one another. Therefore, the intelligent use of these quantities and their calculation as has been outlined would seem to be a formidable armamentarium at the disposal of the bacteriologist, justifying the present day dearth of applications of suggested equations of the growth curve as a whole. Of course, this somewhat indifferent attitude will no longer be tolerable if and when one general theory of the growth curve and its derived equation is proven to have superior merit. The continuing search for such a theory reflected in the recent efforts at application of the thermodynamics of open systems remains a satisfying and legitimate enterprise of biological science.

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## CHAPTER IX

# Enzymes and Bacteria

### THE NATURE OF ENZYMES

As for all other organisms the living processes of bacteria are associated with the activity of enzymes. *Enzymes* are organic catalysts of biological origin and are probably all proteins. Catalysts are commonly defined as substances which while capable of exerting powerful effects on the velocities of chemical reactions are incapable of initiating reactions. Often the reactions catalyzed by enzymes are undetectable in the absence of the enzymes over extremely long periods of time so that for all practical purposes the enzymes may be considered to initiate the reactions. It is also agreed that catalysts are not incorporated into the end products nor are they otherwise rapidly used up in the reactions catalyzed.

While these characteristics are true of enzymes it is in fact desirable to postulate some kind of combination between the enzyme and reactant, or *substrate*, the term applied to the reactant in an enzyme system. The enzyme-substrate compounds are extremely labile and only exist momentarily before breaking down into the regenerated enzyme and products derived from the substrate. In circumstances in which a steady state does not exist or in cases in which the raw materials for synthesis of replacement enzyme are absent, there is noted a small but nonetheless persistent decrease in enzyme activity. This loss is explained not by the enzyme being used up in the reaction catalyzed but rather because of an unrelated, even if intrinsic, deterioration of the enzyme. As a matter of fact, an enzyme will often deteriorate at a lower rate in the presence than in the absence of substrate. The deterioration is probably a reflection of the protein nature of the enzyme, and at the temperatures at which metabolic activity goes on some significant loss of protein by denaturation must be expected. In addition, *in vivo* all enzymes are probably continuously exposed in an environment where numerous and diverse chemical reactions are going on. It would not be improbable that chemically reactive substances like proteins may be degraded by some of these reactions. Possibly some enzymes may be substrates susceptible to the action of other enzymes including those digesting proteins by hydrolytic means.

While an enzyme serves to activate the substrate it does not permanently contribute energy to the system catalyzed. The energy of the enzyme going

into the substrate-enzyme complex is the same as for the enzyme coming out of this combination.

Direct evidence of a combination between enzyme and its substrate has been obtained for a number of systems with many of the details understood in a few cases. For example, catalase has been shown to combine with hydrogen peroxide and other related substrates. Something is known of the rates involved, and complexes thus formed have been studied by means of their absorption spectra. In the course of an enzymatic reaction these complexes are formed and then undergo further reaction returning the enzyme to the system and yielding the decomposition products of the peroxide. In most systems the evidence for formation of an enzyme-substrate complex is not so conclusive. However, at present all enzymatic processes are thought to involve such a step since a number clearly do, and no evidence to the contrary has been obtained in any instance.

Knowing this much one might expect that the properties of the enzyme-substrate system would be quite characteristic of that system and that its nature could be studied by variation in the substrate concentration. Such studies are now commonplace and have proven valuable particularly in elucidating the action of enzyme inhibitors. For the present, however, let us confine ourselves to an analysis of the system of enzyme and substrate.

If one assumes the following reaction where  $E$  is enzyme,  $S$  substrate, and  $ES$  enzyme-substrate complex



then the equilibrium constant  $K_{ES}$  for the first step is

$$K_{ES} = \frac{[E][S]}{[ES]} \quad (2)$$

where the brackets indicate the respective concentrations. Since the concentration of free enzyme  $[E]$  is equal to the total concentration of enzyme  $[E_T]$  less that in the complex one may substitute

$$K_{ES} = \frac{([E_T] - [ES])[S]}{[ES]} = \frac{[E_T][S]}{[ES]} - [S] \quad (3)$$

Ordinarily the desired constant  $K_{ES}$  cannot be evaluated from equation (3) because  $[ES]$  cannot be measured readily, however, other information may be utilized. First the rate constant  $k$  of the dissociation of  $ES$  into  $E + S$  is given by

$$k = \frac{v}{[ES]} \quad (4)$$

where  $v$  is the initial velocity of reaction. Next it should be realized that the maximum velocity  $V$  is attained only when the concentration of sub-



strate is so great that essentially all of the enzyme combines with substrate in the complex  $ES$ . Under these conditions  $[E_T] = [ES]$  and

$$k = \frac{V}{[E_T]} \quad (5)$$

Equating (4) and (5) and solving for  $ES$ :

$$[ES] = \frac{v[E_T]}{V} \quad (6)$$

now substituting (6) into (3) yields

$$K_{ES} = \frac{V[S]}{v} - [S] \quad (7)$$

This equation may be rewritten in the form

$$[S] = V \frac{[S]}{v} - K_{ES} \quad (8)$$

Now since  $V$  and  $K_{ES}$  are constants, equation (8) is that of a straight line and the two constants may be evaluated by plotting  $[S]$  against  $[S]/v$ . The required experimental data, therefore, consist of values of the initial reaction velocity at various concentrations of substrate. This process has been employed in Figure 44.

The magnitude of  $K_{ES}$ , called the *Michaelis-Menton constant* is characteristic of each enzyme-substrate system and indicates the stability of the complex toward its dissociation into free enzyme and substrate. Even more useful is the study of the mechanism of enzyme inhibition in which  $K_{ES}$  plays an important role.

#### NOMENCLATURE

Apart from those enzymes named before universal agreement on nomenclature was reached, enzymes are labeled by the ending *-ase*. They may be named according to the substrate acted on, the substrates representing either a class of compounds or a particular substance:

CLASS OF COMPOUND		SINGLE SUBSTANCE	
Substrate	Enzyme	Substrate	Enzyme
Protein	Protease	Gelatin	Gelatinase
Lipid	Lipase	Lecithin	Lecithinase
Carbohydrate	Carbohydrase	Lactose	Lactase
Nucleic acid	Nuclease	Ribonucleic acid	Ribonuclease

Or the name of the enzyme as in the case of alcoholase may be derived from a product of reaction. This procedure is generally unsatisfactory and

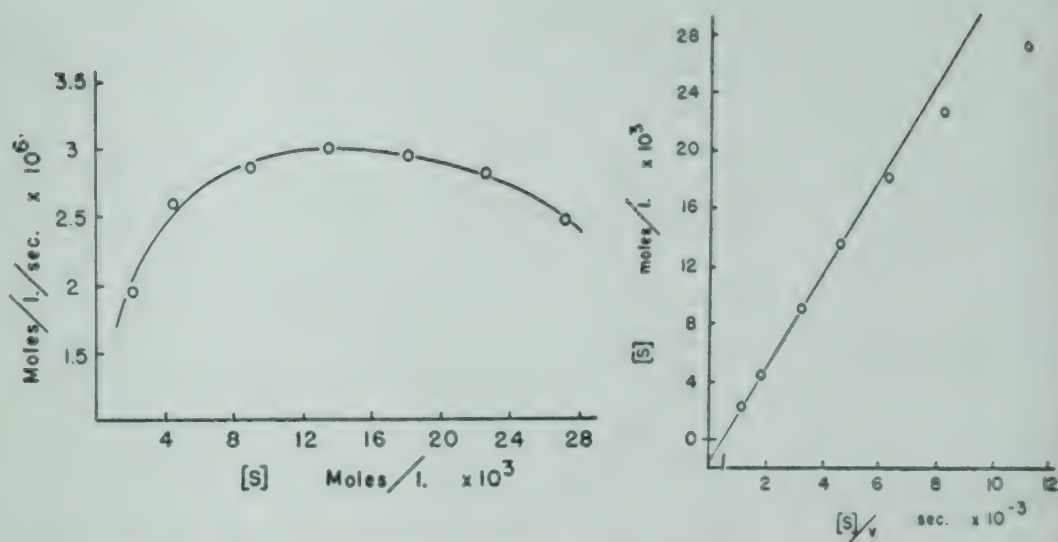


FIG. 44. The effect of substrate concentration on the activity of an enzyme is illustrated with tyrosinase from the mushroom *Psalliota campestris* and catechol.

Part A. Left. Enzyme activity expressed as the initial velocity  $v$  is plotted against substrate concentration revealing a maximum in the activity curve which soon falls away.

Part B. Right. The same data plotted according to equation (8) page 273 portrays typical behavior at low substrate concentrations but a marked deviation at the higher values. It will be noted that the calculated maximum velocity is greater than the observed maximum which reaches only 3.0 moles/liter/sec. If the fraction of total substrate in  $ES$  is small,  $K_{ES} = ([E_T][S])/[ES] - [S]$  (equation (3) page 272). When all of the enzyme is bound in the complex  $[E_T] = [ES]$  and  $K_{ES} = 0$ . If  $[ES]$  approaches zero then  $K_{ES}$  becomes very large, and if half the enzyme is in the complex  $K_{ES} = [S]$ . In this system where  $[S] = 0.0136$  moles/l. at the maximum observed velocity, 90% of the enzyme is combined with the substrate as estimated by means of equation (3).

not a frequently used scheme of nomenclature. A more common practice is to name the enzyme from the nature of the reaction catalyzed, to wit:

Reaction	Enzyme
Splitting of compound by	
Hydrolysis	Hydrolase
Phosphorolysis	Phosphorylase
Rupture of carbon chain	Desmolase
Addition or removal of phosphate	Phosphorylase or dephosphorylase
Oxidation	Oxidase
Reduction	Reductase
Hydrogen transfer	Dehydrogenase
Removal or addition of $CO_2$	Decarboxylase or carboxylase
Hydration or dehydration	Hydrase or dehydrase
Amination or deamination	Aminase or deaminase
Transamination	Transaminase
Isomerization	Isomerase

Since the specificity of an enzyme is closely related to the nature of the substrate, reference to a particular enzyme is more meaningful when the name used is derived from the substrate. Thus to speak of lactase identifies a particular carbohydrase which in fact exists in nature and can only attack lactose. It would be less definite to identify this enzyme, which splits lactose by hydrolysis, by calling it carbohydrase or hydrolase.

#### CHEMICAL NATURE OF ENZYMES

As far as is known all enzymes are globular proteins. They may be simple proteins composed exclusively of amino acids or conjugated proteins. In either case, enzymes from different living organisms acting on the same substrate tend to be antigenically species specific in spite of the fact that no discernible difference may exist in their mode of action. Evidence for this view is supplied by those few cases where it has been possible to compare the antigenicity of purified enzymes obtained from different species.

In the case of the enzymes that are conjugated proteins the non-protein portion of the molecule may be identical for a given enzyme occurring in various species. Thus any antigenic differences in the enzyme possessed by the different species must be attributed to the protein moiety of the conjugated protein molecule. The difference in the protein portion is probably responsible for those variations sometimes exhibited in the enzymatic activity by a given enzyme from different species, differences in stability such as sensitivity to heat denaturation and the optimal conditions for activity such as optimum pH.

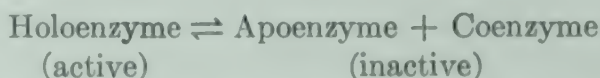
Enzymes such as those discussed above which catalyze a given reaction but differ in some other respect are referred to as *isodynamic enzymes*. They commonly are obtained from different species, and the more distant the relationship between the sources the more different isodynamic enzymes are likely to be. The possibilities for differentiation appear to include all the properties of proteins except of course the reaction catalyzed. For example, the catalytic efficiency, molecular size, optimum conditions, solubility, antigenicity, and in extreme cases the coenzyme or even the presence or absence of a prosthetic group may be different for two isodynamic enzymes.

Whether enzymes from different individuals of the same species are identical or isodynamic is still debatable. The most sensitive tests in use for homogeneity have not been capable of resolving an enzyme preparation derived from a number of individuals, hence the best evidence favors identity throughout a species.

In the case of a conjugated enzyme the protein and non-protein portions of the molecule may be bound together tightly by bonds which can only be split by supplying a large amount of energy. In such a case the nonprotein



portion of the molecule may be referred to as a *prosthetic* group. In other cases the non-protein part of the molecule may be easily separated from the rest of the molecule and is called a *coenzyme*. The coenzymes of proteins are organic compounds, fairly thermostabile, and of low enough molecular weight to be separable from the protein moiety by dialysis through a cellophane membrane. The protein moiety is called an *apoenzyme*. Alone neither the coenzyme or apoenzyme are capable of acting as catalysts for the specific reaction. The complete and active enzyme molecule is called the *holoenzyme*:



Many enzymes are inactive unless metallic ions are added to a system. These inorganic activators of enzymes may be called *cofactors*. In general while one kind of metallic ion is most effective others may also act as cofactors. Thus enzymes activated by magnesium frequently are also activated by manganese and calcium. This situation is true even though under natural circumstances only one of the ions serves as the activator or cofactor. While no case is known in which an inorganic anion is absolutely required for activity, numerous examples of increases in enzymatic activity in the presence of anions have been described.

#### FACTORS INFLUENCING ENZYME ACTIVITY

The factors universally affecting enzymatic activity are the concentrations of enzyme and substrate, the accumulation of reaction products, temperature, pH, and salts.

The velocity of a reaction catalyzed by an enzyme is directly proportional to the concentration of enzyme. At a high concentration of enzyme the activity falls off as the concentration of substrate becomes limiting.

Since the activity of an enzyme is expressed as a rate, it will be apparent from the previous discussion of the Michaelis-Menton constant that the velocity of the reaction depends upon the substrate concentration at low concentrations. As was indicated in this connection, many enzymes possess a maximum velocity of reaction when the concentration of substrate exceeds the quantity necessary to keep the enzyme-substrate complex at a maximal level. However, in a number of systems an unexpected effect is observed. In these cases the rate increases with substrate as anticipated and approaches a maximum. Then with still higher concentrations of substrate the rate diminishes steadily, sometimes very sharply. This situation is illustrated in Figure 44. So far satisfactory explanations of this phenomenon are not forthcoming in systems of this kind in which the inhibitory

substrate concentration is rather low, occasionally an order of magnitude lower than in the instance chosen.

The accumulation of products tends to reduce the velocity of the enzyme catalyzed reaction pointing, of course, to the equilibrium nature of most such reactions. Since the velocity of the reaction is directly proportional to the concentration of enzyme a plot of the rate of accumulation of the product of the reaction should be a direct measure of the concentration of enzyme. However, the reduction of velocity with increasing concentration of the products of reaction limits the useful period for such a determination to the beginning of the reaction when the concentration and influence of the products of the reaction are at a minimum. For this reason it is common to quantitate enzyme concentration from initial reaction velocities.

The velocities of enzyme catalyzed reactions increase with temperature. This rise in activity is reduced at and above the temperature at which significant quantities of the enzyme are heat denatured. At sufficiently high temperatures but little active enzyme remains and the activity diminishes to zero.

An optimal pH exists for the activity of any given enzyme. This pH value is not fixed but varies with the state of purity of the enzyme, its biological source, the temperature, and the nature and concentration of salts present. In view of these variables it is not surprising that the pH values for optimal activity *in vivo* are often not found when measurements are made *in vitro*.

Salts may influence the activity of enzymes in a variety of ways. First, of course, are the specific activations by metals as cofactors and the opposing effect, namely, specific inhibitions by various metallic ions. In addition to these well recognized cases there are others not so commonly considered.

In high concentrations of salt the enzyme may be precipitated or even inactivated thus sharply reducing the velocity of substrate disappearance. Even if losses of enzyme do not occur the alteration of viscosity may modify reactions in which diffusion plays a part. Furthermore, if water enters the reaction a high solute concentration will so reduce the activity of the water as to retard that reaction.

On the other hand salts at all concentrations are adsorbed by protein ions, one sign of electric charge on the protein usually being more affected than the other. This process alters the net charge of the enzyme-protein (see page 186) which may markedly alter the reaction between the enzyme and its substrate. Similarly any process that significantly changes the dielectric constant of the system may modify the charge on the enzyme and hence its activity.



## BIOLOGICAL CLASSIFICATIONS OF ENZYMES

The enzymes may be classified according to the conditions of their biological occurrence. Three such possible schemes will be considered.

## CLASSIFICATION ACCORDING TO SITE OF ACTIVITY

Enzymes may normally be limited to the confines of the cell in which case they are called *intracellular enzymes*. As might be expected, the great majority of enzymes are intracellular. There are, however, some organisms including species of bacteria which quite readily produce *extracellular enzymes*, enzymes normally accumulating in the medium supporting growth.

The extracellular enzymes, like all others, are proteins and thus high molecular weight substances. Their large size poses the problem of how they can be passed through the confining boundaries of the organism without any appreciable loss of all the other physiologically important intracellular molecules. One possibility is that the extracellular enzymes are synthesized at the surface of the organisms and thus need not penetrate the cell wall prior to their release into the medium. A difficulty in this hypothesis is the finding of the same kinds of enzymes intracellularly as occur extracellularly. It must be admitted, however, that this observation need not be considered as decisive proof of the intracellular origin of extracellular enzymes since no one has troubled to, or succeeded in isolating and purifying both types of enzymes for the purpose of a thorough comparative study of their structural identity.

The exclusive origin of extracellular enzymes directly from living bacteria has not been shown clearly. Some bacteria may die even under the most favorable circumstances for development of a culture. Consequently, the accumulation of extracellular enzymes may conceivably be the result of autolysis or leakage of intracellular enzyme from dead bacilli which have lost the selective permeability characteristics of the living organism. We are unaware of the existence of quantitative studies attempting to relate the accumulation of extracellular enzyme to the death rate during active growth. Such investigations would permit unequivocal conclusions as to the importance of accumulation of extracellular enzyme by these non-metabolic processes.

Certain of the observations pertaining to the permeability of membranes as discussed on page 218 may be considered here in connection with the extracellular enzymes. It will be recalled that films are "penetrated" by various materials capable of forming complexes with the film. As described by Rideal, penetration involves incorporation into the film itself resulting in a change in film properties. Undoubtedly those proteins possessing the proper kinds, number, and distribution of chemical groups for formation of a complex with the material of a given film may also penetrate that film



under certain conditions. Such a mechanism could permit the synthesis of an enzyme inside a cell followed by its incorporation into the cell membrane. In this position the enzyme may then possess its normal catalytic activity.

It has been observed that when pressure is exerted on a mixed monolayer film, two layers appear, still in contact with each other, but each made up of only one of the film components. For example, when cholesterol and protein are spread on water a monolayer of lipo-protein complex is formed. If this film is compressed the protein appears as a layer on the water surface overlaid by the cholesterol to which it is still attached. Assuming greater polarity for the medium than for the cell contents, one may conceive that protein appears on the surface of a cell by such a scheme. Once in the surface the protein may then be lost in some way to the medium as extracellular enzyme.

Obviously to be useful such a hypothesis must permit a high degree of specificity since only a minor proportion of the various enzymes in a cell appear in the medium. Admittedly there are many unknowns in the postulated mechanism, and experimental investigation would be difficult. However, directions of study may be deduced assuming the idea to have merit. For instance, the elaboration of extracellular enzyme would depend upon the relative polarity inside and outside the cell. Attempts to correlate the enzymatic activity with dielectric constant might thus be used to check the validity of this portion of the mechanism.

The known extracellular enzymes of bacteria are all catalysts for hydrolytic reactions and include proteases, carbohydrases, nucleases, and lipases. In each case, the substrate is a rather high molecular weight compound which when hydrolyzed by extracellular enzyme yields products of increased solubility in water and of lower molecular weight. The extracellular enzymes may thus be considered important as agents for converting nonpenetrating materials into more readily diffusable and assimilable products.

Since hydrolytic intracellular enzymes, similar if not identical to the extracellular hydrolases, are regularly found in bacteria, it is desirable to know what part these play in the intimate metabolism of the cell. There would appear to be three hypothetical roles from among which it is not possible to choose one as the only true natural role.

For one thing the hydrolytic reactions are reversible so that intracellular hydrolases may serve to activate synthetic reactions thus reversing the reactions accomplished by the extracellular enzymes. This suggestion is not very plausible because the similarity or identity between the intracellular and extracellular enzymes indicates that they ought to have similar equilibrium constants. In this event hydrolysis should be as extensive inside the cell as it is outside, and the small molecular weight products of hydroly-

sis would be favored over their condensation products. However, if the large structures have for any reason a relatively low thermodynamic activity inside the cell then a synthesis may be visualized. In order to reduce the thermodynamic activity it would be necessary to postulate a secondary reaction in which an insoluble compound is formed or from which a stable complex results. In either case the unfavorable equilibrium constant of the original synthetic reactions would be overcome by removal of the product of that reaction. In the formation of enzymes this idea would further require that the secondary reaction must not destroy the enzymatic activity. One can only say that this general scheme is possible but that the severity of the conditions make it seem unlikely.

Still another possibility is that the intracellular hydrolases serve to convert high molecular weight inclusions or insoluble food reserves stored by the cell to assimilable products. Thirdly and maybe most important, the intracellular hydrolases may participate in the maintenance of the steady state characteristic of living processes. In the biological open system there is a continuous two-way flow:



Thus the products of synthetic metabolic processes are not dead ends of flow through the open system. Rather the dynamic state of the system is maintained in part by breakdown processes which follow after the syntheses. As a result, the organism does not accumulate and maintain all the products it synthesizes. While some of these materials may be precipitated in the cell as inclusions from which there is a net withdrawal when flow from the outside universe decreases, there is a continual turnover in synthesized products under all circumstances. This turnover is necessary to maintain the dynamic nature of the biological steady state which depends on the existence of conditions interfering with the establishment of true equilibria. Thus, in part, the intracellular hydrolases by their mere presence may serve to break down synthesized products, a condition which helps make true equilibrium states impossible and contributes to the two-way flow of the dynamic living system.

Measurements of the activity of extracted intracellular enzymes often reveal them to have greater apparent activity than in their *in vivo* location. These findings may be due to a closer approximation *in vitro* to optimal conditions of pH, substrate concentration, solvent concentration, etc. than exist *in vivo*. There is another possibility, namely, the protoplasm may possess natural enzyme inhibitors which are removed by the process of extraction.



The word inhibition suggests something harmful, something non-physiological. It is wise, however, to consider a contrary view for the naturally occurring enzyme inhibitors in the *in vivo* environment. They may serve the useful purpose of moderating the too great activity of some enzymes which must operate in a harmonious complex of reactions. In an integrated network of reactions, desirable results may follow from a moderate pace of activity of the component parts rather than from having individual reactions proceeding near their maximum rates (fig. 45). It may be disruptive to have the velocity of a single reaction unrelated to the naturally

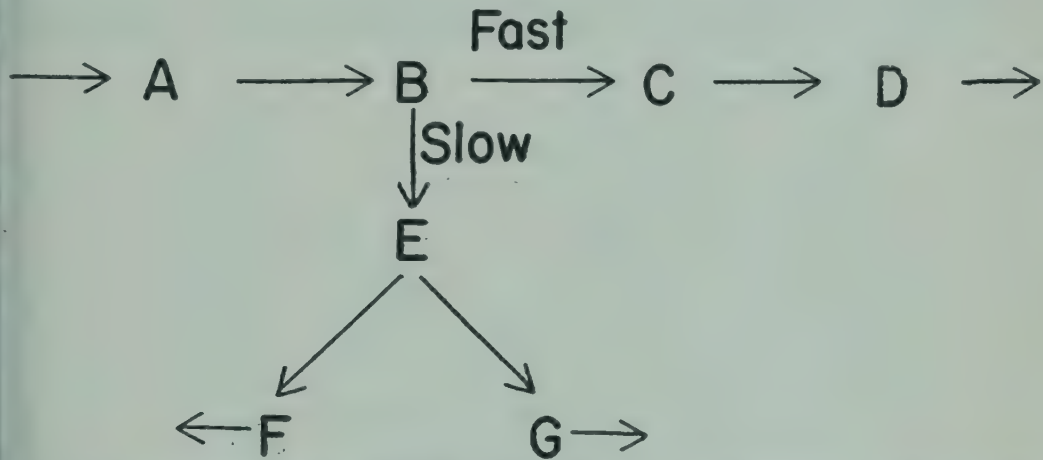


FIG. 45. A hypothetical system of reactions in which mediation by an inhibitor might be important. If reaction  $B \rightarrow C$  is very fast in the presence of enzyme, the concentrations of  $E$ ,  $F$ ,  $G$  etc. may be undesirably small. The presence of an inhibitor for  $B \rightarrow C$  might then increase the quantity of  $B$  reacting to  $E$ .

Such a concept raises another problem; namely, has evolution in general led to the development of inhibitors under such circumstances or to a reduction in the quantity of enzyme?

slower paces of preceding and antecedent reactions. In any case, the pace of any given enzyme reaction must be less than maximal if any preceding reaction in the catenary series leading to the formation of the given substrate is limiting.

#### CLASSIFICATION ACCORDING TO CONDITIONS GOVERNING OCCURRENCE OF ENZYME

The number and kinds of enzymes synthesized by bacteria are characteristic for each species and particular strain of organism. The enzymatic constitution of bacteria is genotypically fixed, nonetheless the genotypic potentialities are expressed only as the environment of the organism provides a suitable substratum for growth and synthesis. The phenotypic or actual enzymatic constitution of bacteria at any given moment including



both the kinds and the absolute and relative quantities of individual enzymes has been found to vary with the nature of the external environment in which the organisms occur. The chemical composition of the environment including pH (fig. 46), the temperature, and the cultural age are all influential variables related to enzyme synthesis by bacteria.

The cultural age of bacterial growth is an expression of the changing conditions in a medium accompanying the growth and metabolic activity. It is these conditions rather than age *per se* which are probably influential in enzyme synthesis. Thus changes in permeability toward substrate, the removal or increase in toxic substances, and the exhaustion of the supply of growth factors are conditions changing with time and may be the factors

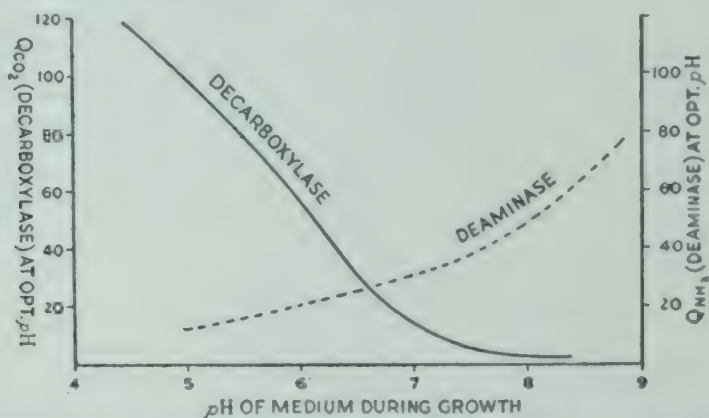


FIG. 46. Variation in glutamic acid decarboxylase and deaminase production by *Escherichia coli* as a function of the pH of the medium during growth.

(From Gale, 1948)

immediately responsible for variations in enzyme synthesis that are associated with aging.

Particularly notable is the presence of specific substrate in the medium as a determining stimulus for the synthesis of some enzymes. Based on this fundamental observation two kinds of enzymes may be distinguished, *constitutive* and *adaptive* enzymes. A constitutive enzyme is one whose synthesis is not dependent upon the addition of its substrate to a medium. In contrast is the adaptive enzyme, an enzyme synthesized in response to the presence in the environment of its specific substrate. It must be re-emphasized that the synthesis of both constitutive and adaptive enzymes is genetically controlled and that a particular kind of enzyme may be either constitutive or adaptive depending on the genotype of a given organism. Table 19 records data to illustrate the basis for the differentiation between adaptive and constitutive enzymes. *Escherichia coli* is shown to be capable of fermenting glucose regularly irrespective of the chemical composition of the growth medium and thus by definition possesses a con-

stitutive enzyme system for this purpose. Yet the same strain will only produce enzyme attacking xylose when growth occurs in a medium containing xylose and similarly will produce enzyme for the fermentation of arabinose only when arabinose is present during growth. The fermentation of these pentose sugars evidently is accomplished by specific, adaptive enzymes.

Under any circumstances the ability to detect an enzyme is limited by the sensitivity of the method used for its detection. It is understood that based on a negative result these techniques do not permit a categorical denial of the absolute presence of an enzyme but only the statement of its occurrence in less than detectable quantities under the experimental condi-

TABLE 19  
*Adaptation by Escherichia coli*

GROWN IN A MEDIUM INCLUDING	SUGAR SUBSEQUENTLY FERMENTED		
	Xylose	Arabinose	Glucose
Arabinose.....	0	+	+
Xylose.....	+	0	+
Glucose.....	0	0	+
No sugar	0	0	+

The method used to collect the above data was simply to grow the organism in the indicated medium, then centrifuge out the growth, and resuspend the washed organisms in a fresh sugar solution and test for the fermentation of the sugar. If the specific enzyme system for fermenting the particular sugar had been synthesized under the growth conditions fermentation would occur. (From Karström, 1930.)

tions. This point is important especially as it bears upon the problem of the mechanism of adaptive enzyme formation. For example, one hypothesis employs the law of mass action to explain the formation of adaptive enzyme in response to the presence of substrate. The enzyme is pictured as existing in a reversible equilibrium with a specific precursor, the equilibrium normally favoring the overwhelming existence of the precursor. As a result, the quantity of active enzyme is too slight to be detectable. The appearance of adaptive enzyme upon addition of substrate is visualized to result from a shift of the equilibrium toward the active enzyme state as the enzyme reacts with substrate. This shift may result in turn in a further synthesis of precursor since its own formation is probably dependent on an equilibrium reaction.

Experimental proof of the above hypothesis has proven difficult, and evidence may be adduced against it. Thus the kinetics of adaptive enzyme



formation are not those to be predicted from the mass law. In addition, some adaptive enzymes are apparently synthesized in the presence of substrate even though conditions for activity of the enzyme are precluded. In such cases it is difficult to visualize a combination of substrate and enzyme, the action necessary for the conversion of precursor to enzyme according to the mass law.

As a matter of fact the kinetics of adaptive enzyme formation have been used to argue for an autocatalytic origin of this type of enzyme, but although the kinetic curves are sigmoid in shape they are not conclusive evidence for this latter view. A sigmoid curve would also result if the individual bacteria introduced into the medium containing substrate varied according to some normal frequency distribution curve in their ability or in the time it took to synthesize the adaptive enzyme.

For some enzymes, which would be labeled constitutive since they occur in detectable quantities in the absence of substrate, large increases are found upon the addition of substrate to the growth medium. With some strains of *Escherichia coli* a 30-fold increase in saccharase activity has been noted when the organisms are grown in the presence of sucrose. The increased enzyme formed as a response to the presence of the sucrose might be labeled adaptive enzyme to distinguish it from the smaller quantities of constitutive enzyme always present in the organism. Such a device assumes a basic difference either in the mechanism of synthesis or the nature of the enzymes produced, views for which no experimental evidence is forthcoming in the case cited.

The presence of specific substrate is not always a necessary condition for adaptive enzyme formation. Examples exist of the production of these enzymes in response to the presence of a product of the reaction they catalyze. Thus a case has been recorded of the production of lactase in the presence of galactose, a product of the hydrolytic cleavage of lactose. A similar example is the production of enzyme attacking creatine when creatinine, an end product of the reaction, is used in the growth medium in place of the creatine. In still another case high concentrations of casein hydrolysate were found to substitute for nitrate as substrate for the adaptive formation of nitratase.

On the other hand the continuous presence of specific substrate is not always necessary for the production of adaptive enzyme. An interesting example is the production of penicillinase by *Bacillus cereus*. Unadapted bacilli need merely be exposed for a brief time to penicillin at a temperature at which growth does not occur, and when washed and transferred to a medium free of penicillin and incubated they will produce considerably greater quantities of penicillinase than control cultures whose inocula have never been exposed to penicillin. The amount of penicillin adsorbed by



the exposed bacilli is considered to be so small as to preclude the possibility of any penicillin being carried over into the second medium free of penicillin. This possibility is also made unlikely by the fact that any small quantities of penicillin initially present by reason of transfer along with the inoculum would be rapidly destroyed by the first quantities of penicillinase produced in the developing culture.

The observations cited can be used to defend the thesis that a differentiation between adaptive and constitutive enzymes is one only of convenience and serves merely to separate enzymes as to functional types. No fundamental difference in the basic synthetic mechanism for their production need exist. The logically acceptable view has been offered that these two types of enzyme represent only the extreme cases of variability of enzyme synthesis in response to factors in the environment. Constitutive enzymes would vary least and adaptive enzymes most. Whether this is a true view or not of the real nature of the differences in enzyme formation, the preservation of the distinction as a scheme for identifying enzymes is of scientific value. It serves to focus attention upon the adaptive enzyme systems as unique practical examples for the study of the fundamental biological and biochemical problems of protein synthesis.

Investigations of the nature of the biological synthesis of proteins have been almost uniquely difficult. In fact no ideas on the subject are sufficiently dignified by the existence of evidence to deserve the title of theory. Ordinarily one would endeavor to study the formation of enzymes since these are more easily detected and estimated than those proteins without known pronounced catalytic activities. However, the formation of the constitutive enzymes is not readily dissociated from cell division, and the great variety of changes thus occurring more or less simultaneously makes it exceedingly difficult to distinguish the processes leading to the synthesis of the enzyme. Since adaptive enzymes do form in resting cells in the presence of substrate but in the absence of growth, their study offers the most promise.

The exploratory studies now visualized include attempts to follow exchanges among the cellular components that may be correlated with appearance of enzymatic activity. In this connection radioactive phosphorus studies show that pentosenucleic acid reactions are involved. Other attempts have been directed toward the isolation of inactive compounds intermediate in the formation of adaptive enzyme. Addition of such intermediates to adapted resting cells might be expected to increase the total activity. Such approaches as these may eventually lead to the details of protein synthesis and maintenance.

At the present time bacteria, yeasts, and other fungi are the organisms which best show adaptive enzyme formation. The likelihood of detecting adaptive enzymes in multicellular organisms is reduced by the existence of

the cells in an internal environment that is regulated and varies in no way as radically as the environments in which microorganisms are placed.

Another interesting fact is that the great majority of adaptive enzymes described are hydrolases or in any case are enzymes which act upon the raw materials that enter into the early stages of cellular metabolism. Of course, once a primary substance has been introduced and acted upon metabolically its products are inevitably present as intermediate metabolites. As long as life processes go on intermediate metabolites must be present whether their ultimate source is from exogenous nutrient or of endogenous origin from cellular substance and reserves. Thus enzymes acting upon intermediate materials will always be present, and it has not been possible to know whether they are produced in response to the presence of their substrates or not. This is true not only for the reason cited but also because the large variety of starting materials for living processes are metabolically reduced to fewer and common products of catabolism. Thus the deprivation of a particular nutrient does not guarantee the non-appearance in the organism of all the intermediate metabolites associated with the metabolic utilization of the nutrient.

The production of bacterial adaptive enzymes usually requires an exogenous source of nitrogen. This is true even when adaptive enzyme formation can take place in "resting cell" or nonmultiplying suspensions. Actually, most examples of adaptive enzyme formation by bacteria require active growth and proliferation of the organism in the presence of the specific substrate.

After adaptive enzyme has been formed the removal of substrate will generally result in a loss of the enzyme. In some cases this appears to take place by the mere process of simple dilution of the original quantity of enzyme as growth in the culture proceeds without further synthesis of enzyme. In other cases the enzyme may be disposed of catabolically and serve as raw material for further protein synthesis. One transfer into a medium free of substrate is commonly sufficient to eliminate the adaptive enzyme. However, there are reports that multiple transfers through media containing substrate may result in a much slower loss of adaptive enzyme activity upon subsequent transfers through substrate-free nutrient media. Even in these cases only a few transfers are required before all adaptive enzyme activity becomes undetectable. Hinshelwood has reported an exceptional case in which a given strain will not deadapt after many subculturings. Other investigators, however, have interpreted Hinshelwood's data to mean that a mutant containing a constitutive enzyme was selected.

The synthesis of adaptive enzymes seems to require the expenditure of energy. The evidence for this requirement is based chiefly on the inhibition of adaptive enzyme production by chemical poisons specific for respiratory



processes. The poison may act to uncouple the energy transferring mechanism from anabolism. Since poisoning a respiratory mechanism may concomitantly interfere with the accumulation of intermediates serving in synthetic processes, the mere demonstration of inhibition of respiration is not definitive proof of an energy requirement for adaption. Although clear proof of an energy requirement is difficult, that in itself does not

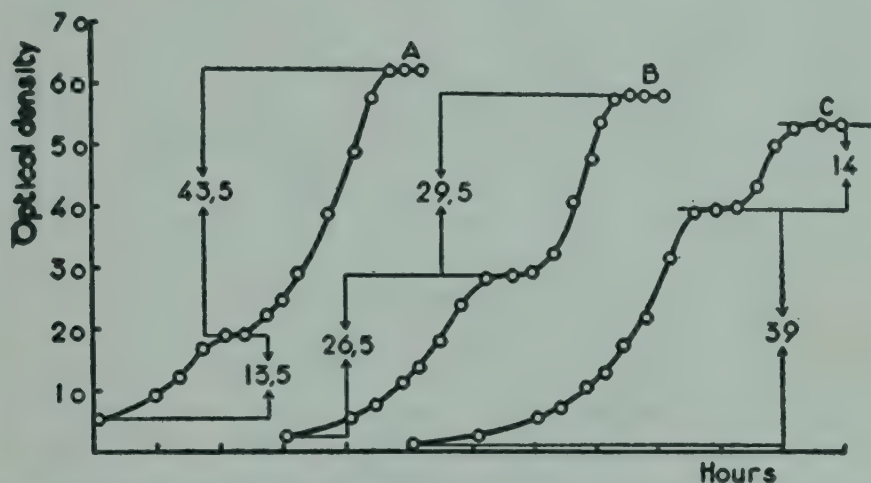


FIG. 47. Illustration of the diauxie phenomenon using *Escherichia coli* grown in a synthetic medium with glucose and sorbitol in limiting quantities serving as the carbon sources.

Media: A. Glucose 50  $\mu\text{g.}$  per ml; sorbitol 150  $\mu\text{g.}$  per ml.

B. Glucose 100  $\mu\text{g.}$  per ml; sorbitol 100  $\mu\text{g.}$  per ml.

C. Glucose 150  $\mu\text{g.}$  per ml; sorbitol 50  $\mu\text{g.}$  per ml.

Growth during first cycle utilizes glucose whereupon a lag phase ensues following the complete utilization of the glucose. The second cycle following this lag represents the growth associated with the utilization of the sorbitol. The lag phase between cycles represents time for adaptation for the utilization of the sorbitol. The figures between the arrows indicate the total growth corresponding to each cycle. Note that the proportion of total growth occurring in each cycle is a function of the ratio of glucose to sorbitol.

(From Monod, 1942)

detract from the logical idea that the synthesis of the new molecules will undoubtedly require an input of energy.

Competition between adaptive enzymes during their formation and between an adaptive and constitutive enzymes has been found. The first clear-cut demonstration of interactions in the synthesis of adaptive enzymes was afforded by the discovery of the phenomenon called diauxie. *Diauxie* is the division in the development of a culture containing a mixture of substrates into cycles of growth separated by a lag phase (fig. 47). Thus *Escherichia coli* in response to the presence of both glucose and sorbitol grows in a nutrient mixture of these substances utilizing only the



glucose at first. An adaptive enzyme for the utilization of the sorbitol does not appear until the glucose has been consumed. A lag phase intervenes between the periods of the growth cycle during which the different sub-

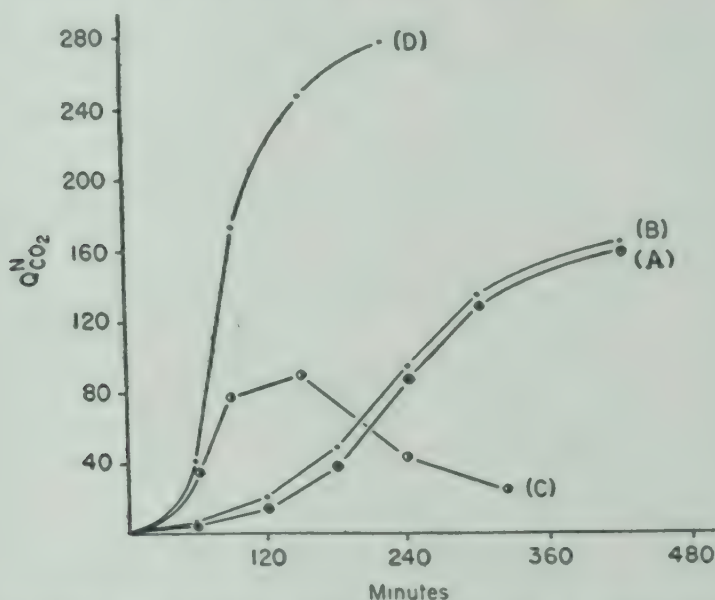


FIG. 48. Competition in adaptive enzyme formation to different substrates. Example illustrated is the case of adaptive enzyme formation by a resting cell suspension of yeast.

Curve: A. Galactozymase activity during simultaneous adaptation to galactose and maltose fermentation.

B. Galactozymase activity during adaptation to galactose fermentation alone.

C. Maltozymase activity during simultaneous adaptation to maltose and galactose fermentation.

D. Maltozymase activity during adaptation to maltose fermentation alone.

From these data it is evident that the synthesis of the adaptive enzyme galactozymase is not affected by the presence of maltose while the formation of the adaptive enzyme maltozymase is inhibited by the presence of galactose. In other words, the adaptive synthesis of galactase competes successfully against the adaptive synthesis of maltozymase.

(From Spiegelman, 1950)

substrates are utilized and represents the time it takes for the adaptive enzyme to be produced. The portion of the total growth contributed by the utilization of the individual catabolized substrates is a function of the ratio of the substrates. The competition in the formation of adaptive enzymes in mixtures of substrates can also be shown for those organisms capable of synthesizing the enzymes in the absence of growth both in the cases where an exogenous source of nitrogen is and is not required (fig. 48).

Whether or not the competition in enzyme formation is evidence for the concept of enzyme synthesis drawing upon a common pool of precursor material has not been settled. Nor has the direct interconversion of adaptive enzymes been established. Thus the disappearance of a given enzyme has not been found to bear any regular mathematical relation to the amount of any new enzyme which may appear at the time the original enzyme is disappearing. Such a finding might be expected if the substance of one enzyme could be converted directly into the substance of another enzyme.

Because of the probable specific and differing natures of the chemistry of the individual protein apoenzymes the possibilities discussed may be viewed critically and with skepticism. Any protein that can be catabolized in the dynamic living system may be a potential supply of individual amino acids, larger structures, and even of individual atoms for assimilation into other and entirely unrelated proteins. In this connection the intriguing problem of how the organism manages to synthesize only particular enzymes from among its potential enzyme make-up when given a limited supply of utilizable inorganic or organic sources of nitrogen must be considered. A beginning has been made with this problem, the data for which provide still another basis for the convenient designation of types of enzyme, a system which will now be discussed.

#### CLASSIFICATION ACCORDING TO DISPENSABILITY TO THE ORGANISM

If bacteria are grown in media with limiting quantities of assimilable nitrogen, a decrease in the total nitrogen of the organisms is observed. The per cent by weight of nitrogen in the dry matter of *Escherichia coli* has been found to vary from about 13 per cent to a lower limit of 6.5 per cent over the concentration range of 0.8 per cent to a minimum of 0.004 per cent ammonium sulfate in an otherwise complete nutrient medium. Thus the developing bacterial culture responds to limiting concentrations of building material not only by a decrease in total population but also by decreasing the amount of the building material incorporated into the structure of the individual bacterium.

Under the circumstances one of two things can happen in regard to enzyme synthesis. The amount of all enzymes per individual can be equally reduced, or the production of some enzymes can be maintained near normal levels at the sacrifice of other enzymes. Apparently in the natural situation the latter circumstance is the rule. It thus becomes possible to distinguish enzymes on the basis of their preferential synthesis under conditions limiting the supply of nitrogen.

Those enzymes synthesized preferentially have been called *indispensable* enzymes while *dispensable* enzymes are those synthesized in relatively small quantities per cell under conditions of limited nitrogen supply. The

dispensable enzymes may be looked upon as participating in the less critical metabolic processes. Contrariwise the indispensable enzymes are those without which the organism is unable to remain viable. Teleologically the organism may be said to make a choice, when a choice must be made, that favors the continued existence of the essential metabolic functions at the sacrifice of less critical activities. Actually this can be reduced to the more

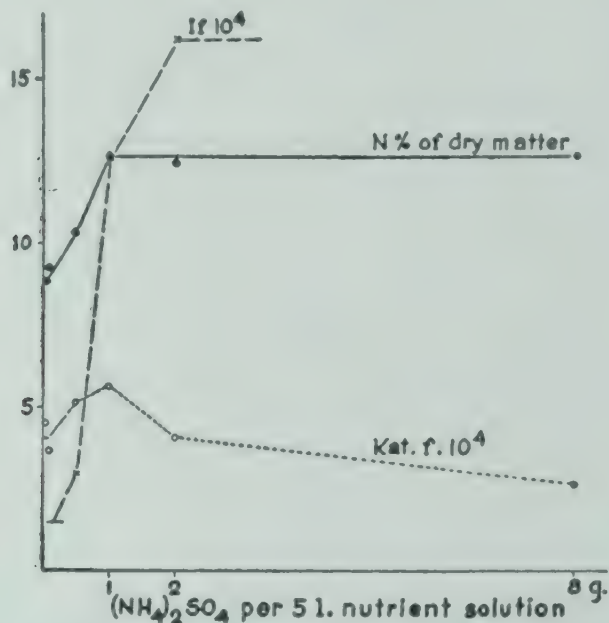


FIG. 49. Catalase and saccharase activity and nitrogen content of *Escherichia coli* grown in media with limiting concentrations of  $(\text{NH}_4)_2\text{SO}_4$  as the source of nitrogen. *Kat.f* represents activity units of catalase while *If* represents units of saccharase activity. The data illustrate the relative lack of dependence of the synthesis of catalase on the nitrogen composition of the organism and the contrary dependence of the synthesis of saccharase on the nitrogen content.

(From Virtanen & De Ley, 1948)

scientific statement that in the long run only those organisms survive whose metabolism is best fitted for life in the uncertain natural environment.

There is another possibility. The nitrogen may go to an enzyme because metabolism takes it there, not because that enzyme is indispensable. Death may result from failure at *one* critical step and one cannot tell whether other active systems are critical or not. On the other hand this argument cannot be raised in connection with the disappearance of the dispensable enzymes. In this instance if the enzyme is lost but the organism still lives, one must admit that the dispensable enzyme is not critical in any absolute sense. In any case one may say that only those bacteria possessing a flexible metabolism involving both the dispensable and indispensable types of enzyme have persisted into modern times.



Bacteria grown in the presence of limiting quantities of a nitrogen source do not show any large decrease in the rate of either their exogenous or endogenous respiration. In addition, catalase appears to be an indispensable enzyme. Since there is no clear notion as to what essential metabolic role this enzyme plays this finding ought to stimulate renewed interest in the problem of its natural function. Most curious is the observation that intracellular proteolytic enzymes, substances whose true importance has not very much concerned modern students of intermediate metabolism, are of the indispensable type. The dispensable type of enzymes have been found to be the carbohydrases (fig. 49) and adaptive enzymes in general.

What are the minimum activities essential for the support of life? This philosophical question may now be considered subject to attack by studies of the nature of indispensable enzymes and the metabolic activities in which they participate.

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## CHAPTER X

# Physical Factors Affecting Bacteria

### SURFACE TENSION

The surface tension of pure water at ordinary temperatures is about 72 dynes per  $\text{cm}^2$ . The natural presence of nutrients in water or their addition causes a drop of the surface tension to the range of 45 to 65 dynes per  $\text{cm}^2$ , and within this range most bacteria seem to do well. Experimental manipulation of media so as to further reduce the surface tension has led to the common impression that surface tension influences the growth, multiplication, and morphology of bacteria and has a lesser effect on the gram reaction, motility, sporulation, and spore germination.

With intestinal organisms such as the enterococci and gram negative coliform bacilli, media of rather low surface tension will support growth. Possibly, such growth may be the result of an evolutionary adaptation of these organisms since their natural environment in the gastro-intestinal tract is rich in surface active agents, particularly bile salts. There are, however, other bacteria, notably the pneumococci and meningococci, which appear to be unable to grow at surface tensions much below 50 dynes per  $\text{cm}^2$ . With these organisms a reduced surface tension can actually result in lysis. The mechanism of this lysis has not been elucidated.

Since it is difficult if not impossible to prepare a nutrient medium with a surface tension higher than that of water, there are no bacteriological data available on the response of bacterial metabolism and growth to conditions of high surface tension. The presence of the bacteria themselves in media of high surface tension would tend to reduce that surface tension by reason of excretion of surface active metabolic substances.

Among the more manifest effects of the growth of bacteria at low surface tension is the tendency for the average adult size, particularly the length, to increase and for growth in broth to be dispersed for organisms ordinarily showing pellicle formation. While changes in multiplication and metabolic rates have also been described, it is more difficult to associate these changes with surface tension *per se*. There are several reasons for this complication.

Surface tension measurements are only an indirect measure of the surface force actually operating on the bacterium, namely the tension of the medium-bacterial surface interface. Unfortunately there are no known means for measuring this interfacial tension. A further difficulty arises



because a substance must be added to the medium in order to reduce surface tensions, and the presumed surface effects may actually be specific chemical effects dependent on the nature of the added substance.

While it appears logical to attribute effects to surface tension when two unrelated chemicals reducing the surface tension to equal values produce similar results, even this interpretation may be too simple a view. As has been pointed out before, the accumulation of substances at an interface is related to the effects solutes have on their mutual solubilities. Therefore, addition of substances reducing the surface tension of solutions to the same extent does not necessarily result in interfaces of identical chemical composition unless each solute happens to affect the solubility of all other solutes present in the solution in exactly the same way. It is doubtful in the case of bacteriological media which include a diversity of constituents and products of growth of the bacteria that this condition can be met. Hence, unless the chemical composition of interfaces as well as the interfacial tensions can be shown to be identical, we can never be sure surface energies alone are responsible for the events observed.

Still another consideration involves changes in the physical arrangement of organisms in a growing mass brought about by a change in surface tension. Reduction of interfacial tensions causing clusters of cells to separate into single cells or resulting in dispersed growth instead of pellicle formation may be followed by changes in metabolism or multiplication rate. But are these latter changes the result of the manipulation of surface tension or the change in the physical arrangements of the mass of organisms? Possibly cells in clusters do not exchange nutrients and metabolic end products with their environments as rapidly as do single cells, and certainly bacilli dispersed in the body of a medium are not growing in a relatively well aerated environment as would be true when they are growing as a pellicle directly exposed to the atmosphere.

From these few considerations it is evident that the subject of surface tension as it relates to bacterial activity is a more complex one than a superficial glance at the problem might indicate. The difficulties outlined may explain the relative lack in the scientific literature of information on the subject and the practically total absence of reports of definitive experiments. This deficiency exists in spite of the fact that for the purpose of diagnostic bacteriology there is no lack of useful media of reduced surface tension for the selective growth from mixed cultures and identification of particular species. An example of the latter is the use of a nutrient medium containing sodium ricinoleate to reduce the surface tension to 40 dynes per  $\text{cm}^2$ , where *Lactobacillus acidophilus* will grow but where *Lactobacillus bulgaricus* will not grow.

## OSMOTIC PRESSURE

Like that of other organisms the metabolic activity and morphology of bacteria appear to be affected by osmotic pressure. With a given strain there exists an optimum osmotic pressure for activity and a tendency for the organisms to increase in size and to assume a more nearly spherical shape when exposed to lower osmotic concentrations. The optimum generally consists of a fairly wide range of values, but different species are not all alike in this respect. The osmotic pressure responses of bacteria are readily modified by processes of adaptation. Thus while sudden changes in environment from a high to a low osmotic concentration, particularly with organisms from cultures in the early stages of development, may result in a loss of viability, gradual changes or successive subcultures in media of osmotic concentrations varying by small increments often result in no notable changes in viability. In view of this adaptability there seems to be little justification for the idea that "physiological" salt solution is the truly physiological solution for bacteria. Finally, sporulation and spore germination are not demonstrably affected by variations of the osmotic pressure of the environment within the range permitting growth.

Including both gram negative and positive types, bacteria growing in natural habitats of high osmotic concentration, such as sea water, salt lakes, and fruit juices may require media of high osmotic concentration for their successful isolation in the laboratory. While most of these species can be adapted to growth in media of lower osmotic concentration, others are obligately osmophilic, an *osmophilic* organism being one requiring an environment of high osmotic concentration. If such organisms are associated with environments of high salinity they are more often spoken of as *halophilic* while those occurring in high concentrations of sugar are said to be *saccharophilic*.

The salinity of the oceans is about 3.5 to 4 per cent, and marine halophiles have been described which require anywhere from 2 to 15 per cent salt. The Dead Sea with a saline content of about 29 per cent is known to harbor obligate halophiles which will not grow at concentrations of salts below about 13 per cent. Practically no information is available to suggest how the metabolism of these latter organisms can be dependent upon such an unusual growth condition.

A complete theory of halophilism must account for two phenomena, the dependence upon high salt concentration for growth, and the resistance to concentrations of salts harmful for other bacteria. There is no satisfactory hypothesis at present accounting for the nature of the dependence of the growth of halophiles on high salt concentrations. On the other hand, three



possible hypotheses have been proposed to explain how halophiles resist high salt concentrations:

1) The enzymes and proteins of halophiles are assumed to be unusually resistant to salting out. Few data have been collected to test this notion.

2) Since salts diffuse with difficulty through hydrophobic barriers the surface membranes of halophiles have been regarded as rich in, or as composed predominantly of hydrophobic substances. However, the surfaces of halophiles do not seem to be unusually hydrophobic in nature, nor are the acid-fast bacteria, which as a group are high in lipids, generally more salt tolerant than non-acid-fast species. Therefore, this hypothesis is not attractive.

3) By the expenditure of energy the halophiles might maintain a low intracellular salt concentration against a concentration gradient. This hypothesis is supported by a recent finding that a nitrataase of *Micrococcus halodenitrificans* is salt sensitive in the cell-free state and salt tolerant in its normal intracellular state. However, this enzyme is inhibited in resting cell suspensions at high salt concentrations by the addition of respiratory inhibitors. The implication of these findings is that interference with exergonic metabolism prevents the expenditure of energy necessary for the maintenance of a low intracellular concentration of salt in the face of a high extracellular concentration of salts.

In the preservation of foods by salting or canning in concentrated sugar solutions advantage is taken of the fact that an upper limit of osmotic concentration exists for the growth of common organisms. The minimum concentrations of salts and sugars which may be safely employed for preservation of particular foods is dependent on the opportunities for the natural contamination of the foods with osmophilic organisms. Invariably much higher concentrations by weight of sugars (50–70%) are needed than of salts (10%). In comparing the relative efficiency of sugars and salts there must be taken into account the smaller molecular weight of salts and their capacity to ionize, both factors increasing the relative osmotic concentration. High concentrations of solutes frequently may have specific chemical toxic actions apart from their influence on osmotic pressure.

This observation raises the problem of what criteria can be used to separate specific chemical effects from osmotic effects. In general, it has been thought that an osmotic effect is proven when dissimilar substances have the same effects at the same osmotic concentrations. With sugars it is not difficult to obtain data on the limiting osmotic pressures for growth with different species, but this is not true of salts since the limiting osmotic concentrations supporting growth vary, and may vary widely, with different salts. The same type of observation has been made for the salt requirements of halophilic organisms. In the case of an obligate anaerobic halophile



isolated from salted anchovies  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Li}^+$ ,  $\text{Mg}^{++}$ , and  $\text{Ca}^{++}$  supported growth in decreasing minimum total ionic concentration.

Studies of the relationship of osmotic concentration to the intermediary metabolism of bacteria are practically nonexistent. Some work has been done with the luminescent reaction of the luminous marine bacteria. Luminescence of these organisms is reduced by diluting the sea water or by exposure to equivalent hypotonic solutions of sucrose. Luminescent flashes are reduced both by hypotonicity and hypertonicity.

During the growth of bacteria, changes are to be expected in both the absolute and relative concentrations of impermeable and dialyzable particles inside and outside the bacterial organism though little work has been reported on this problem. One may only state with certainty that during the phase of adjustment and the early exponential growth phase the osmotic concentration of intracellular substances is at a maximum. This, of course, is responsible for the elevated turgor pressure normally associated with actively growing cells. The protoplasm at this stage of cultural development is actively synthesizing new substances and probably is unusually rich in low molecular weight intermediates involved in respiration and serving as building blocks for the synthesis of the larger carbohydrate, lipid, nucleic acid and protein molecules.

### SONIC ENERGY

The study of the effects of sonic energy on bacteria is a field of developing interest for its intrinsic merit as a scientific problem, as a means for attacking problems of the structure of bacteria, and for the isolation of molecular and macromolecular components of these organisms. Exposure of bacteria to sonic energy in the regions of supersonic and ultrasonic frequencies results in readily notable changes. *Audible sound* is generally considered to be restricted to frequencies below about 9000 cycles per second. *Supersonic* waves are defined as those in the frequency range of 9,000 to 200,000 cycles per second although the term is sometimes used more generally to denote any frequency greater than 9,000 cycles per second. The term *ultrasonic* is usually applied to frequencies above 200,000 cycles per second.

In recent years an increasing number of satisfactory sonic generators have become commercially available to the laboratory worker. These are all transducers for the conversion of electrical energy to sonic energy and are either magneto-strictor oscillators or piezo-electric generators. The term *transducer* is applied to any device actuated by power from one system and supplying power to a second system.

In the magneto-strictor oscillator a stack of nickel laminations is set into longitudinal oscillation by surrounding it with a long coil through which alternating electric current flows. Since nickel has the property of contract-

ing when subjected to a magnetic field, the nickel laminations will contract and expand under the influence of the alternating flux of the electromagnetic field induced by the flow of alternating current through the coil. The natural vibration frequency of nickel limits the usefulness of the magnetostrictor devices to the supersonic range.

The piezo-electric generators utilize the property of certain crystals when connected with an alternating electric current to be compressed during one half of the cycle and to be expanded by the same amount in the other half of the cycle. Quartz crystals have been found to be most satisfactory. The

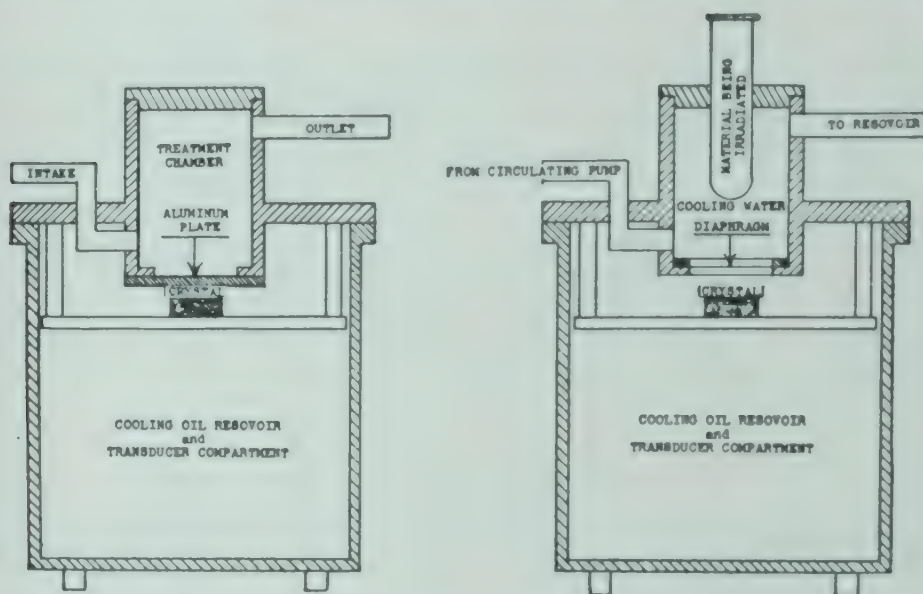


FIG. 50. Diagram of an ultrasonic generator  
(Courtesy of Ultrasonic Engineering Co.)

natural mechanical frequency of quartz crystals is such as to make them suitable for generating vibrations in the ultrasonic range.

The end result of exposure of bacteria to high frequency sound waves is death and lysis. Measured by these effects the efficiency of the devices in use seems to be less dependent on the frequencies of the sound waves employed than on their amplitude. For the most effective killing of bacteria the instrument is operated at the highest possible wattage or energy output and at the frequency corresponding with resonance. The student will recall that objects commonly have a natural vibration frequency. The sonic generator then is said to operate at resonance when the sonic frequency corresponds to the natural frequency of the nickel laminations or crystal resonator and conversely to be in dissonance when the frequencies are out of harmony with the natural frequency. In Figure 51 an experiment is

illustrated showing the importance of operating the sonic generator at resonance.

The killing of bacteria by sonic energy is accompanied by an actual rupture of the organism and dispersal of intracellular contents. The effect can be followed by counting procedures as well as by a decrease in the turbidity of bacterial suspensions. The decrease in turbidity accompanying the exposure may be preceded by an initial increase. This preliminary

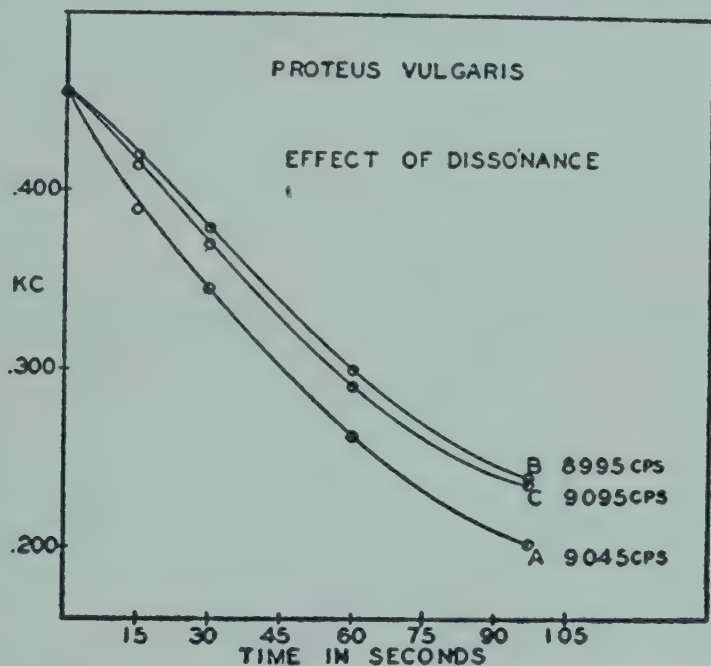


FIG. 51. Turbidity curves of a culture of *Proteus vulgaris* when treated with super-sonic energy from the sonic generator operating at resonance and dissonance. Resonance for the generator in the case illustrated was about 9045 cycles per second. The ordinate expresses turbidity, hence the lower curve represents maximum killing and corresponds to maximum power output from the generator.

(From Shropshire, 1947)

change has been shown to be due to a dispersal of chains or clumps of organisms into individual cells resulting in a rise of turbidity which does not begin to fall off until significant numbers of the organisms are lysed.

Bacteria differ greatly in their ability to resist sonic energy. Some species are very susceptible, suspensions of an organism like *Neisseria gonorrhoeae* being easy to sterilize while with many others it is impossible to kill 100 per cent of the organisms with any degree of regularity. Bacterial spores are quite resistant being unaffected in most cases. While it is not known how some individual organisms can resist destruction indefinitely, it has been suggested that bacteria are small enough so that some may accumulate



at the nodes of transverse vibrations and thus escape the tearing effects of sonic energy. It is probable that the most destructive position for organisms is between the nodes of vibrations.

The mechanism of the killing of bacteria by sonic energy is not well understood, and the quantitative data suggest no obvious explanation. The per cent mortality is independent of the initial concentration of organisms (Table 20). Although the death rate may simulate a monomolecular process at the beginning of exposure, and for a subsequent period of time, it does not remain so for the entire course of the exposure. This change is reflected by the fact that in most cases the slopes of logarithmic survivor curves change after the fraction of survivors becomes small.

Organisms suspended in menstrua of high viscosity or of high surface tension are less susceptible to sonic energy. These findings may be inter-

TABLE 20

*Data illustrating the lack of dependence of the percentage of mortality of Klebsiella pneumoniae exposed to ultrasonic waves on the initial concentration of organisms*

INITIAL COUNT PER ML	PER CENT VIABLE CELLS AFTER TIME IN MINUTES OF ULTRASONIC TREATMENT				
	10	20	30	40	50
92,300,000	32.5	10.4	2.65	0.95	0.53
36,000	46.4	10.1	2.98	1.91	0.88

(From Hamre, 1949.)

preted to support the theory of cavitation and consequent rupture of the outer membranes of bacteria as the cause of death.

Unlike the case for larger organisms direct observations on individual bacteria of the events occurring during exposure to the sound waves have not been reported. Any discussion of the nature of the biological effects of sound waves must therefore be drawn from experience with other organisms. These studies have suggested several possible causes of death and lysis of cells, namely, violent agitation of the intracellular contents, disruption of the normal colloid structure of protoplasm, and cavitation.

With large cells exposure to high frequency sound waves is accompanied by an active stirring of the cellular contents, but this phenomenon is less prominent the smaller the organism and may not occur in bacteria. Exposure of colloids *in vitro* may result in flocculation, liquefaction of gels, or emulsification, the effects being greatly dependent on the nature of the colloid. Such changes occurring in cellular colloids may conceivably be responsible for the biological effects of high frequency sound. However, no direct evidence for their occurrence in bacteria has been reported.

*Cavitation* depends upon the formation of minute bubbles of dissolved gases upon exposure of the solutions to sonic radiation. The mechanism of cell destruction by cavitation is thought to involve the rapid striking of the cellular boundaries by innumerable gas bubbles appearing in the cavitated liquid immediately surrounding the cell. Thus the violent motion of external gas bubbles during cavitation is the crucial factor rather than the expansion of gases within the cell.

The collision of cells or their contact with particulate matter cannot be the cause of the rupture of the cellular membranes. The addition of particulate matter such as carbon particles and sand has been shown not to enhance the biological effects of the supersonic radiation. Evidence for cavitation as the cause of death is supported by the following observations depending upon effective means for simultaneously reducing both cavitation and the biological effects of supersonic energy: (1) application of a sufficiently high external pressure of gas, (2) replacement of dissolved gases in a medium by more soluble gases, and (3) evacuation of gases from the suspending medium before the application of the sound waves.

### SOLID SURFACE

All other things being equal the growth of bacteria is unaffected by the volume of the medium. However, in growing bacteria the tendency is to choose similar shaped containers of various sizes to fit the volumes of media to be employed, and under these circumstances there often has been the impression that a greater population per unit volume grows out in the smallest volumes. On the other hand a critical analysis reveals that the volume of medium is unimportant. Since the surface areas of similarly shaped receptacles exposed to unit volume of medium will be greater in containers of decreasing capacity, the effects noted are due to this difference, the relative amount of solid surface of the receptacle exposed to the medium rather than to the volume of medium. In Figure 52 this has been confirmed by growing bacteria in fixed volumes of medium held in different shaped vessels and by adding varying quantities of glass beads to a fixed volume of medium in order to increase the solid surface. The degree of aeration, and surface area of medium exposed to air, has been controlled in these experiments in order to eliminate the possible influence of variations in oxygenation.

The favorable effect of solid surfaces is evident only in dilute nutrient media. As a rule, nutrients must be less concentrated than 25 parts per million (.0025%). The effect of solid surface on bacterial growth is prominent in the case of sea water and other raw waters which naturally contain only low concentrations of nutrients (fig. 52). An ecological aspect of this

phenomenon is the fact that bacteria are a part of the plankton, the mass of free and passively floating microscopic life of the sea. The bacteria are most abundant in those natural waters rich in suspended particulate matter, both living and non-living.

The effects of the addition of fixed amounts of materials such as glass beads to increase the solid surface are more pronounced the more homogeneous the distribution throughout the body of the medium. In other words, any arrangement which increases the opportunity for bringing all portions

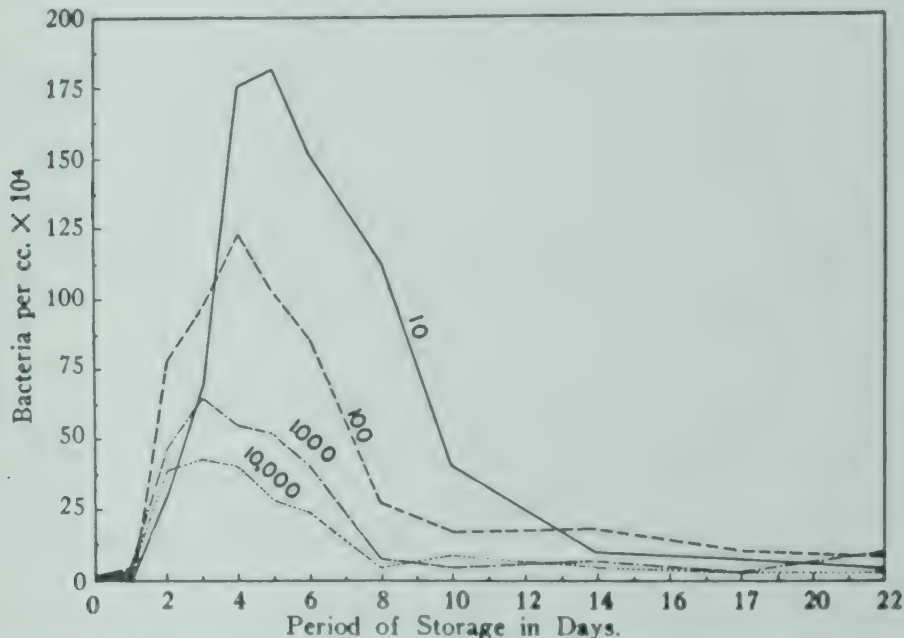


FIG. 52. Influence of the volume of sea water in containers of similar shape on the multiplication of bacteria in stored sea water. The numbers on the curves represent volumes of sea water in an appropriate container about  $\frac{5}{6}$  full.

(From ZoBell & Anderson, 1936.)

of the medium into close proximity to the solid surface enhances the beneficial effect of the solid surface.

The size of particulate matter is also important. Particles larger than the bacteria are beneficial, but smaller particles may be without effect or actually harmful. The latter may be due to the fact that particles smaller than the bacteria may be adsorbed to the bacteria and reduce the effective surface area of the bacteria available for direct communication with the nutrient fluid.

Some bacteria are *periphytes*, i.e. they grow attached to solid surfaces and may be obligately periphytic. Such organisms may secrete mucilaginous substances and become glued to solid surfaces or they may adhere by means



of a morphologically differentiated structure, a *holdfast*. Solid surfaces would serve such organisms as resting places for their growth.

The favorable effect of solid surfaces for the growth of more typical bacteria is thought to be due to concentration of nutrients by adsorption inasmuch as the deposition of organic matter on solid surfaces from dilute solutions has been shown experimentally. It has been pointed out previously that a minimum limiting concentration of nutrient probably exists for growth. The presence of solid surfaces in media of low nutrient concentration would raise the concentration in their immediate vicinity. Bacteria coming into this environment by chance or by reason of any force which attracts or holds them to the solid surface thus would be exposed to more favorable concentrations of food than would exist in the bulk of the medium. As a result their growth would be enhanced. The total population supported by the medium would tend to be determined more by the increased concentrations of foods available near the solid surfaces than by the concentrations in the bulk of the liquid or the concentration calculated from the total food available in the entire volume of the medium. Such an explanation is in accordance with the importance of solid surfaces only when in dilute media and not in situations where the limiting factor for population growth is other than a minimum concentration of food. Finally, the concentration of extracellular hydrolytic enzymes and their products of reaction at solid surfaces might be important for bacteria dependent on extracellular hydrolysis for rendering nutrients into assimilable forms.

The above explanation is also supported by observations that indicate a reduction in growth by factors interfering with the accumulation of bacteria at the solid surface. Thus the addition of surface tension depressants which tend to disperse bacteria has a retarding effect as does coating the solid surface with thin films of hydrophobic greases. In these cases fewer of the individual bacteria remain in the immediate environment of the solid surface with its higher concentration of food. An interesting case of the influence of solid surface on growth in distilled water is presented in Table 21.

### RADIATION

Like all other living forms bacteria are influenced by radiation, the effects being generally harmful except for the organisms possessing pigments that make possible the metabolic utilization of some radiations. While visible radiation may be harmful the magnitude of the incident exposure required for damage is very much larger (about 100,000 times) than for radiations of smaller wave length (Table 22). Apart from any thermal effects wavelengths greater than those of the visible spectrum such as infra-red and Hertzian rays are not generally considered to be harmful. Most investigators have

reported these radiations to be harmless when precautions were taken for preventing a rise in temperature.

TABLE 21

*The effect of talc on the growth of Escherichia coli in distilled water*

DAY	NUMBER OF ORGANISMS GROWING IN DISTILLED WATER				
	Boiled	Boiled with new rubber tubing	Boiled with clean rubber tubing	Boiled with cleaned new rubber tubing plus talc	Boiled with talc
0	380	380	380	380	380
2	0	540,000	0	800,000	1,000,000
4	0	100,000	0	750,000	440,000
7	0	80,000	0	250,000	67,000

The experiment recorded in the table was stimulated by the observation that distilled water passed through new rubber tubing supported some growth but not when passed through old rubber tubing. It was found that this was due to the fact that talc is used as a surface dressing for rubber, and the talc is lost as the tubing is used and washed in the laboratory. Talc acts as an absorbent of gases from the air (probably  $\text{CO}_2$  and  $\text{NH}_3$ ) and renders these gases available as nutrients for the growth of the bacteria. If the talc is separated from the water containing the bacteria by a cellophane membrane the bacteria do not grow. This indicates the talc must be in intimate contact with the water in which the bacteria are to grow. Other substances which can be used in place of talc to show the recorded effect are: asbestos, barium hyposulfite, barium sulfide, barium sulfite, calcium hyposulfite, calcium phosphate, ferrous phosphate, ferrous silicate, kaolin, kielselguhr, magnesium silicate, magnesium dioxide, permutit, silica, silver sand, soil, unglazed porcelain, zirconium silicate.

(From Bigger and Nelson, 1941.)

#### THE NATURE OF THE ABSORPTION OF ENERGY ASSOCIATED WITH RADIATION

Radiation is defined as the propagation or transmission of energy through space. The propagation may occur by means of wave motion or by means of atomic or subatomic particles moving at great velocities and set in motion by the action of electric fields or by ejection from radioactive substances. When radiant energy strikes some material object it may be absorbed thus involving the interaction of the energy with matter. The energy available for absorption is governed by a fundamental law relating the quantum energy propagated by the radiation to the product of its frequency multiplied by the Planck constant:

$$E = h\nu = h \frac{c}{\lambda},$$

where  $h$  is the Planck constant,  $\nu$  the frequency,  $c$  the velocity of light and  $\lambda$  the wavelength of the radiation.

If the quanta have energy equivalent to less than 1 electron volt ( $1.59 \times 10^{-12}$  erg) their absorption will leave the electronic structure of atoms unaffected. In such cases kinetic energy is imparted to the absorbing molecules and only thermal changes will result. Absorption of these radiations may lead to an increased rate for typical chemical reactions but is unlikely to result in any changes in the kinds of chemical reactions taking place.

Irradiation characterized by absorption of quanta of energy greater than one electron volt will have a different effect on matter. In these cases the electronic configuration of atoms results from raising of outer orbital or

TABLE 22

*Effects of the 3500 to 4900 Å and the 2180 to 2950 Å regions of radiation*

	3500-4900 Å	2180-2950 Å
Shape of killing curve (log survival ratio/energy)	Threshold type	Approaching straight line
Energy (incident) for 50% survival ratio	Approximately $2 \times 10^8$ ergs/cm <sup>2</sup>	$5 \times 10^2$ to $10^3$ ergs/cm <sup>2</sup>
Temperature coefficient	1.7-2.2	1.1
Sublethal effects appear	Before any organisms are killed (in threshold part of killing curve)	After 60 to 90% of organisms are killed
Toxicity of certain salt solutions can be recognized	At once after irradiation	In 600 minutes at 32°C

(From Hollaender, 1943.)

valence electrons to a new energy level. Such an excitation results in a more chemically reactive substance, and molecules possessing such an atom or atoms are spoken of as *activated*. The activated molecule has only a short life (order of  $10^{-6}$  second) at the end of which time absorbed energy is lost by one of several means (see also fig. 53):

- 1) fluorescence
- 2) re-emission of a quantum of energy of about the same wavelength
- 3) transfer of energy to another molecule or photosensitization
- 4) degraded to heat in which case the kinetic energy of all the molecules in the system is increased.

When the absorption has an energy greater than about 5 electron volts for the quantum, orbital electrons may be ejected from atoms. This is the case with ordinary ultraviolet light (5-100 electron volts) and for radiation of still smaller wavelengths (x-rays, 10,000-1,500,000 electron volts). The



result is the conversion of the absorbing atoms and molecules into ions. The ejected electrons are subsequently picked up by other molecules so that ionization resulting from radiation involves the production of *ion pairs*. Radiations giving rise on absorption to ion pairs are referred to as *ionizing radiations*.

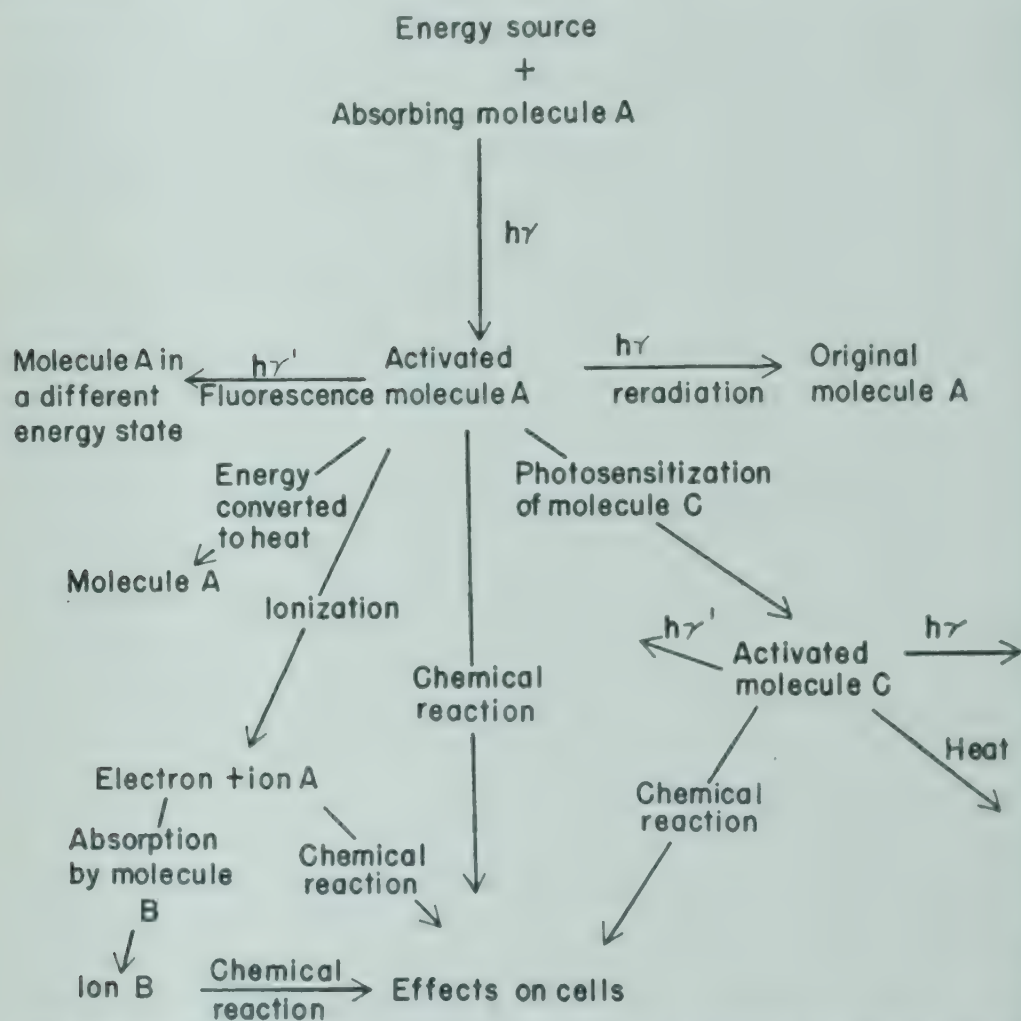


FIG. 53. A schematic representation of the general pathways by which radiation may affect biological systems.

#### BIOLOGICAL EFFECTS OF RADIATION

Ultimately the harmful effects of radiation upon living organisms must be correlated with particular chemical reactions. Exactly what these reactions are is not known. Since exposure of cells to ionizing radiations has been found to result in an increase in the cytoplasmic pentose nucleotide, the suggestion has been made that the breakdown of nucleic acids by ionizing radiations may result in the accumulation of harmful concentra-

tions of nucleotides. Interestingly, the injection of large doses of pentose nucleotides into mice has been followed by systemic effects resembling those induced by excessive doses of x-radiation.

In the case of bacteria the following suggestions have been made to explain death from both ionizing and non-ionizing radiation:

- 1) Inactivation of enzymes.
- 2) Production of poisons by decomposition of cellular material (nucleic acids, proteins, lipids, carbohydrates) or as end products of unusual chemical reactions made possible by ion pair formation.
- 3) Lethal mutations.

If the first two mechanisms are operative some functional activity of the bacteria must be inactivated and, presumably, many molecules would have

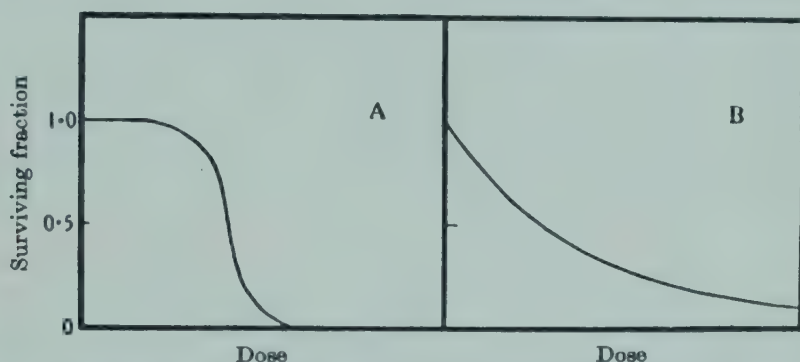


FIG. 54. Survival curves expected for a cumulative type of action (A) and for a single ionization or single ionizing particle type of action (B).

(From Lea, 1947)

to be changed. In such a case a threshold dose of irradiation would exist. Since the loss of a few molecules or a fractional part of the intracellular enzyme would not completely interrupt a functional activity, e.g. respiration or the synthesis of proteins, the bacterial organism would not die until a large number of molecules or the greater part of an essential enzyme were altered. This would take time, so that after initiation of the irradiation a lapse in time would be expected before the organisms showed damage. This lag would be the reflection of a threshold dose, the amount of radiation necessary before a biological effect could occur (fig. 54).

If a lethal mutation were the basic mechanism of death by irradiation, only one gene or one molecule would need to be affected. In this case a uniform holistic response might be expected, and from the very moment the irradiation of a culture begins some bacteria should die (fig. 54). This mechanism of death has a great deal of evidence in its favor.

Still another way in which radiation may be harmful is by the alteration or production of poisons in the medium. Although this action is known to

occur it does not exclude the possibly concomitant direct bactericidal and mutagenic actions of radiation. Bacteria can be studied in a non-nutrient medium, or better still they can be washed free of medium, exposed to irradiation *in vacuo* in a dehydrated state, and then plated out in testing for lethal effects. Since radiation is found to be effective under these conditions the destructive action is exerted directly upon protoplasmic structures, and death could be caused in the same way when medium is present.

When bacteria are in contact with medium the lethal effects of exposure to radiations may be due to the combined action of the radiation directly on the organisms and on the medium. Both ultraviolet and x-ray irradiation have been shown to produce bactericidal substances of unknown chemistry from a variety of carbohydrates (including agar) and oils. In addition, hydrogen peroxide is an end product of the irradiation of water and has been shown to produce mutagenic chemical agents from a variety of common nutrient substances including adenine, uracil, tryptophane, and tyrosine. Thus in order to be certain of the direct biological actions of radiation it is necessary to treat dried organisms in the absence of all non-bacterial materials.

### *Ionizing Radiation*

A most attractive theory of the nature of the bactericidal action of ionizing radiation is the lethal mutation, or *target theory*. The target theory postulates that the bactericidal effect is due to a single effective ionization and, therefore, to a change in a single molecule. It is scarcely conceivable that the alteration or destruction of a single molecule could have serious consequences for an organism unless that molecule is concerned with a genetic mechanism. Analogy with the genetics of other organisms suggests the essential molecule to be of the nature of a gene which yields a lethal mutation when altered.

The silhouette of an object or of a bacterium through which an ionizing particle of radiation passes is called the *target*, and the actual passage of an ionizing particle through a target is described as a *hit*. Absorption of ionizing radiation then may be visualized as hits or ion pair formations within the targets traversed by the radiations. The target size may be deduced from knowledge of the spatial distribution of the ionizations and the number of ionizations per unit volume of exposed object to one roentgen of any radiation. Target diameters for mutation in the fruit fly *Drosophila* are listed in Table 23. The dimensions of the target vary with the nature of the ionizing radiation, but all are of the same order of magnitude, namely, within the expected range for protein and other macromolecules. Thus if single ionizations can be shown to result in mutation, the implication is clear. Change in a single molecule (nucleoprotein?), probably a gene, is



responsible for the biological effect. The data in favor of this view are that survival curves of irradiated bacterial populations are exponential (fig. 55), that the bactericidal effect is dependent only on the dose and not the in-

TABLE 23  
Target diameter for mutation

	HARD X-RAYS	SOFT X-RAYS	NEUTRONS	$\alpha$ -RAYS
Relative dose for equal yield of mutation.....	1.00	1.30	1.45	3.44
Target diameter (in $\mu$ ).....		4.4	9.0	6.6

(From Lea, 1947.)

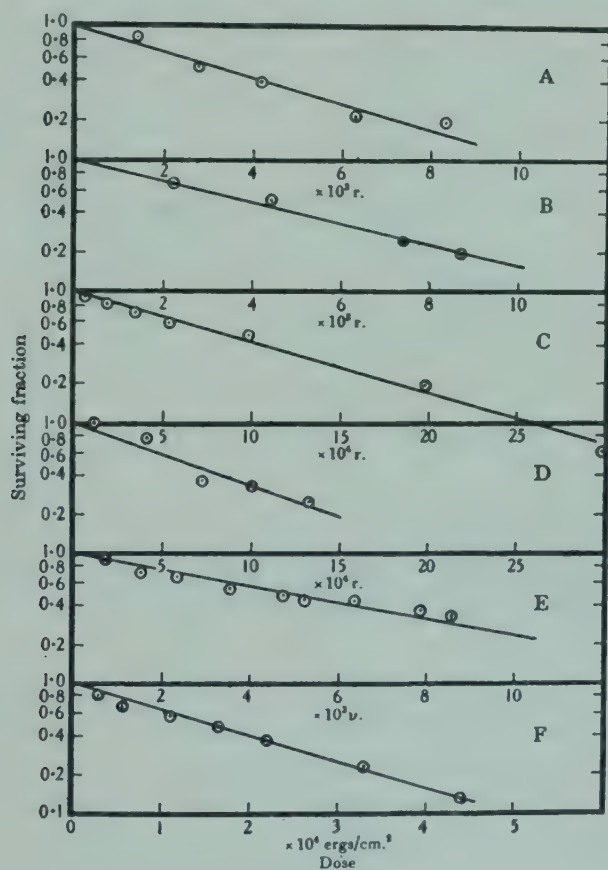


FIG. 55. Survival curves of irradiated bacteria.

- A. X-rays (1.5 Å). *Salmonella aertryke*.
- B.  $\alpha$ -rays. *Escherichia coli*.
- C.  $\beta$ -rays. Spores of *Bacillus mesentericus*.
- D.  $\gamma$ -rays. Spores of *Bacillus mesentericus*.
- E. Neutrons on *Escherichia coli*.
- F. Ultraviolet light (2803 Å). *Bacillus megaterium*.

(Data compiled by Lea, 1947)

tensity of irradiation (Table 24), and that the efficiency or effectiveness of radiations per ionization decreases for the more densely ionizing radiations. These data will now be considered briefly.

The transformation of a single molecule into one or more products is known as a monomolecular or first order reaction. The rate of such a reaction is expressed by the equation:

$$\ln (m - x) = -kt + \ln m \quad (1)$$

where  $m$  represents the initial molar concentration of the reacting species,

TABLE 24

*Showing that the mean lethal dose of radiation is independent of intensity*

RADIATION	ORGANISM	INTENSITY	M.L.D.*
		$r/\text{min}$	$r$
$\alpha$ -rays	Spores of <i>Bacillus mesentericus</i>	$9.84 \times 10^3$	$2.3 \times 10^4$
		$6.12 \times 10^4$	$2.6 \times 10^4$
X-rays (8 Å.)	Spores of <i>Bacillus mesentericus</i>	$6.24 \times 10^3$	$1.5 \times 10^4$
		$6.02 \times 10^4$	$1.2 \times 10^4$
		$4.70 \times 10^5$	$1.7 \times 10^4$
X-rays (0.15 Å.)	<i>Escherichia coli</i>	65	$5.6 \times 10^3$
		209	$5.7 \times 10^3$
Ultraviolet light (2536 Å.)	<i>Escherichia coli</i>	$\text{ergs/cm}^2/\text{sec.}$	$\text{ergs/cm}^2$
		$1.2 \times 10^2$	$7.5 \times 10^3$
		$3.1 \times 10^3$	$8.5 \times 10^3$
		$6.4 \times 10^4$	$8.5 \times 10^3$

\* M.L.D. is the mean lethal dose in roentgen units. It is the dose reducing the survivors to a fraction  $e^{-1} = 37\%$  of the initial number of exposed organisms.

(Data compiled by Lea, 1947.)

$x$  the molar concentration reacting in time  $t$ , and  $k$  the velocity coefficient. Since the equation is one of a straight line, plotting the logarithm of the concentration of actual reacting molecules against time yields a straight line, the slope of which represents the velocity of the transformation, and the intercept on the  $y$  axis represents the logarithm of the initial concentration of reactant.<sup>1</sup> When an equivalent semilogarithmic survival curve of

<sup>1</sup> Since first order reactions are fairly widespread it seems useful to review the derivation of the kinetic equation for a typical system of this class.

The number of survivors after exposure to lethal radiation is the initial number of individuals less the number killed during the time of exposure. For a fixed density or intensity of radiation the number of organisms killed in a given time is proportional to the number of organisms present as long as all the radiation is not absorbed. In

bacteria exposed to radiation is plotted (the log of the number of survivors against time) a straight line is obtained. Thus the killing of bacteria by ionizing radiation resembles a first order reaction (fig. 55).

The analogy is clear, but in and of itself that does not prove the relationship of the mechanism of the bactericidal action to a monomolecular change. There is always the possibility that the presumed exponential curve is actually the result of the existence of a population with a skew or non-symmetrical distribution of sensitivity to radiation among its individuals. This possibility decreases as the actual experimental points on a curve fit the theoretical exponential curve more closely since the necessary degree of skewness would increase and become biologically more improbable. The corroboration of other evidence makes the exponential survivor curve a potent argument in favor of the target theory.

The bactericidal effect of radiation is independent of intensity and dependent only on the dosage (Table 24). By *intensity* is meant the roentgens of ionizing radiation applied per unit time, while *dosage* refers to the total roentgens to which the organisms are exposed. If a single molecular transformation were the cause of death this lack of dependence on the intensity factor would be expected. Each and every ionization caused by a hit has the same probability of being effective and the probability is independent of time. The first hit on any one all-important molecule should result in death independent of the time at which the hit occurred and of preceding and subsequent hits on other molecules.

A single hit causes a number of ionizations but if death is caused by the other words, the quantity of radiation absorbed depends only upon the number of individual targets, and the greater the number of hits the greater the extent of reaction. This general concept is the fundamental characteristic of first order reactions.

A small decrease in the concentration of organisms in a short time is proportional to the concentration of organisms. Expressed in the notation of calculus

$$\frac{-dc}{dt} = kc \quad (2)$$

where  $\frac{dc}{dt}$  represents a very small change in concentration during a short interval and the negative sign indicates that the change is a decrease.  $k$  is the proportionality constant and  $c$  the concentration. If this expression is integrated between limits

$$-\ln c_1 + \ln c_2 = k(t_1 - t_2) \quad (3)$$

or

$$\ln c_2 = -k(t_2 - t_1) + \ln c_1 \quad (4)$$

When  $t_1 = 0$  then  $t_2$  becomes  $t$ .  $c_1$  is the original concentration, and the concentration at time  $t$  is  $c_2$  which may be written as the original concentration less the quantity reacted. Thus equation (4) is seen to take the form of equation (1).



ionization of a single indispensable molecule then all other ionizations resulting from the same hit are without effect. This situation holds even if a single hit resulted in ionizations of several indispensable molecules because the ionization of the first indispensable molecule acted upon would result in death. Subsequent indispensable molecules hit would not have a visible biological effect on an organism already irreparably changed. As a result the bactericidal efficiency of radiation in terms of deaths per ionization would be less for those radiations producing the greater number of ionizations within the target. With vegetative forms of bacteria this expectation is realized and is supporting evidence for the target theory. However, with bacterial spores no evident relationship exists between the density of ionization and the bactericidal efficiency per ionization, an exceptional behavior which remains unexplained.

An interesting consequence of the target theory is the opportunity it affords for estimating the fractional part or volume of the organism which is irreparably changed when hit by quanta of ionizing radiation. If all portions of a bacterium were equally sensitive to radiation then the first hit irrespective of its location within the organism would result in death. Thus in an irradiated population the number of organisms dying per unit time should be equal to the number of organisms absorbing one or more quanta of energy. Physical measurements of the absorption of radiation by bacteria reveal that the death rate is always lower than this maximum value. Obviously the finding means that not all hits on a bacterium result in death. Therefore, only a certain portion of the substance of the organism is considered to be sensitive to the radiation. Only hits within a radiosensitive portion can be effective, and the total intracellular space within which hits result in death has been called the *sensitive zone* of bacteria.

The size of the sensitive zone is easily estimated. The irradiation of the bacilli is homogeneous in distribution, all portions of the organisms have the same opportunity to be hit. With objects as small as bacteria the ordinary ionizing radiations penetrate uniformly to all parts of the living system, so that hits are randomly distributed throughout the organism. Necessarily then, the fractional part of an organism occupied by the sensitive zone must be equal to the fraction of the absorbed quanta or hits which is lethal. The fraction of hits resulting in the death of bacteria is about one in twenty though the exact figure varies with particular organisms. Therefore, about five per cent of the total volume of the bacterial cell is occupied by the sensitive zone. Since the target size for a particular radiation is known, the volume of the sensitive zone divided by the target size is the total number of sensitive targets within the bacterium. It has already been pointed out that these target sizes correspond to the dimensions of macromolecules like proteins, nucleic acids, polysaccharides. If the sensitive tar-

gets represent single and indispensable molecules or genes, their number is obviously a measure of the total number of genes possessed by the organism (about 250 in one strain of *Escherichia coli* studied).

Since the sensitive zone of a bacterium appears to have about the same dimensions as some inclusion bodies staining like nuclear material the sensitive zone has been considered to represent the nuclear apparatus. It should be emphasized, however, that the evidence of the target theory does not reveal whether the individual targets making up the sensitive zone of the organism are organized into a single unit or whether they are dispersed throughout the organism. The mere existence of a sensitive zone does not prove the presence of a morphological nucleus.

### *Ultraviolet Irradiation*

It requires about 100 times more energy to kill bacteria by ultraviolet irradiation than with x-rays. In addition there is no systematic relation of the wavelength to the bactericidal efficiency, rather the incident energy required is least between 2600 and 2700 Å. Usually there is correspondence between the absorption in the ultraviolet region by nucleic acids and proteins and the lethal effectiveness of the radiation. Consequently, the notion that ultraviolet radiation acts by reaction with nucleic acid and/or denaturation of proteins is attractive. The exact chemistry of these reactions is unknown, but they do not require oxygen because ultraviolet light is effective against bacteria both under anaerobic conditions and in the absence of hydrogen peroxide. The lethal mutation theory as applied to ultraviolet irradiation while favored by some authors is denied by others. Examples of survivor curves which are both logarithmic and non-logarithmic have been reported.

It has become increasingly clear within recent times that estimates of the bactericidal efficiency of ultraviolet light are greatly influenced by the cultural conditions in the viability test used after the exposure of the organisms. The chemical nature of the test medium, whether it is used in a solid or liquid form, the temperature of incubation, and the time elapsing between irradiation and plating have been described as affecting the counts obtained. Manipulation of these variables has been found to change the shape of survivor curves, a result which tremendously complicates any theoretical analysis of quantitative data. Furthermore, these variables do not influence different kinds of bacteria in the same way. Even within a single species (*Escherichia coli*) different strains have been found to be differently affected. Such observations suggest a multitude of mechanisms whereby ultraviolet irradiation damages cells.

Since the bactericidal consequences of ultraviolet irradiation are influenced by conditions after the exposure has taken place, the process of



injury cannot be irreversible. The realization that recovery is possible then becomes a great stimulus to research on problems of the therapeutics of irradiation. The bacterial system provides practical experimental material for research in this field at the level of cellular physiology and biochemistry.

*Photoreactivation*

A most interesting phenomenon of recovery from ultraviolet radiation injury is *photoreactivation*, the recovery of ultraviolet inactivated cells by their exposure to visible light (Table 25). The most effective wavelengths for photoreactivation are longer than 5100 Å in the yellow portion of the spectrum. The reactivation is proportional to the time, intensity, and the temperature of exposure to visible light, but illumination before exposure to ultraviolet irradiation does not produce reactivation.

TABLE 25  
*Photoreactivation in four microbial species*

	STREPTOMYCES GRISEUS	ESCHERICHIA COLI	PENICILLIUM NOTATUM	SACCHAROMYCES CEREVISIAE
Dark survival*.....	$2.1 \times 10^{-6}$	$4.5 \times 10^{-6}$	$5.5 \times 10^{-4}$	$1.0 \times 10^{-5}$
Light survival†.....	$6.6 \times 10^{-1}$	$1.2 \times 10^{-1}$	$2.5 \times 10^{-1}$	$1.0 \times 10^{-3}$

\* Fraction of cells surviving in suspensions kept dark after ultraviolet irradiation.

† Fraction of cells surviving in suspensions illuminated with reactivating light after the ultraviolet irradiation.

(From Kelner, 1949.)

*Inhibition of Bacterial Division*

Death of bacteria by irradiation does not always occur immediately. An exposed bacillus may multiply after irradiation and the progeny be unable to multiply. Much more energy is required to cause immediate death than death following division of the exposed organism. Irradiation may also cause a temporary delay of division, the duration of which increases with the dose. As a result irradiated bacteria may grow to unusual sizes before dividing (fig. 56). Delay of division and death at resumption of division cannot be due to the same kind of injury differing only in degree, for the two phenomena vary in different ways with the type of radiation, the dose rate, and temperature of exposure.

TEMPERATURE

A universally influential variable in biological processes is heat. The temperature range within which protoplasm can survive and metabolize is only a small segment of the temperature range possible for the universe



extending from a state of nearly no kinetic energy of matter ( $0^{\circ}\text{K}$ ) to an upper limit of about  $3,000,000,000^{\circ}\text{K}$ . Matter probably ceases to exist in the form of atomic and molecular particles near this latter temperature at

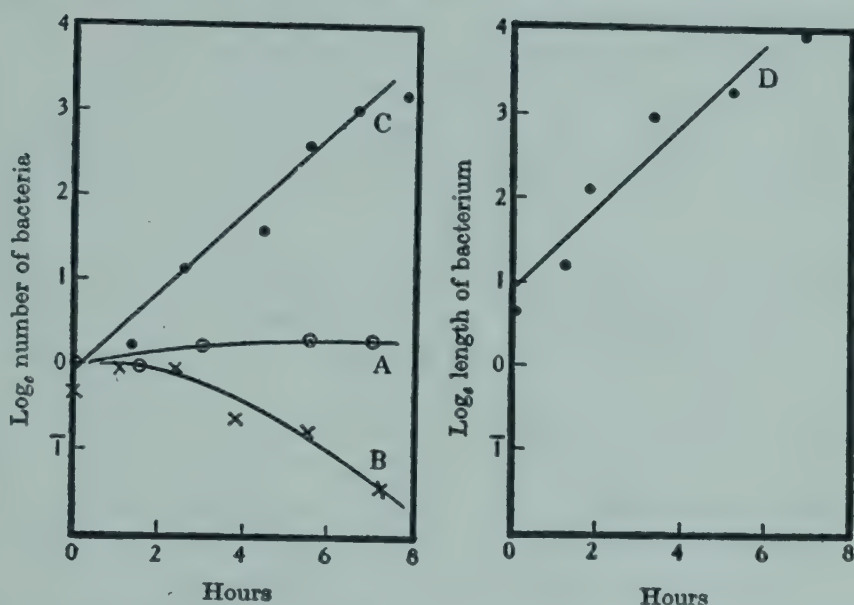


FIG. 56. Inhibition of division in *Escherichia coli*, by continuous irradiation with  $\gamma$ -rays at 35 r per minute.

A. Total count in irradiated culture.

B. Viable count in irradiated culture.

C. Total combined length of all the bacilli in the unirradiated culture.  
(Ordinate same as for D)

D. Total length of the longest organism in the irradiated culture.

Note that the slopes of curve C and D are practically equal. This indicates the rates of growth in the irradiated and in unexposed cultures are alike, but that the division of the bacilli is inhibited. The number of organisms in the irradiated culture is not increasing (A) but the individual size is increasing (D). The best estimate of length as a measure of growth rate after irradiation depends upon individual organisms instead of the population as a whole to avoid the inclusion of dead cells. To include measurements of the length of dead cells would underestimate the true growth rate.

(From Lea, Haines, & Coulson, 1937)

which the density of the energy in the system would be at least equal to the density of the matter.

The upper limit of life as we know it is set by the stability of the indispensable molecules making up organisms. Since insufficient information is available about the composition of cells it cannot be predicated that a given temperature will lead to change of a known key molecule and thus kill a given cell. However, some broad generalizations can be drawn indicating the upper thermal limits expected of any life form.

First it must be borne in mind that many reactions require appreciable times in order to be brought beyond the threshold stage at which biological effects occur. It may thus be that short exposure at a relatively high temperature will not be lethal. Yet allowing the system to come to equilibrium at a lower temperature will permit passage of the time required for the reaction to proceed to the point at which fatal changes take place. Realizing that kinetic effects are important and may account for short periods of existence under unusual circumstances, let us consider the possible chemical effects of comparatively extended heating at somewhat elevated temperatures.

Ordinary chemical bonds are commonly regarded as quite stable to heat, and many compounds remain unmodified at high temperatures. Sodium chloride boils at  $1413^{\circ}\text{C}$  but eventually decomposes when the temperature is raised still higher. Most compounds are not so stable as this. Ordinary covalent bonds are broken at much lower temperatures. For example, methane at  $400^{\circ}\text{C}$  breaks down into carbon and hydrogen to the extent of 14 per cent at equilibrium. Thus insofar as carbon-hydrogen bonds are indispensable, heating to the neighborhood of  $400^{\circ}\text{C}$  will be lethal. Naturally in protoplasm there are stronger bonds present including practically all of the multiple bonds of organic compounds and some single bonds like oxygen-hydrogen. Among the more common weaker single bonds are those between carbon and carbon, carbon and oxygen, carbon and sulfur, and carbon and nitrogen. The latter is perhaps the weakest covalent bond encountered in protoplasm and reduces the theoretical maximum survival temperature considerably since it is only a little over half as strong as the carbon-hydrogen bond.

Other still weaker bonds whose importance in protoplasm may be anticipated but is less certainly established than the foregoing are the so-called hydrogen bonds. These bindings have bond energies from 1.3 to 8.2 kilocalories (kcal.) per mole depending upon the atoms involved as contrasted with values of 87 and 49 kcal. per mole for C—H and C—N respectively. The very low energy values permit reactions involving these bonds to occur at rather low temperatures, and any such lethal reactions will probably limit the temperature survival range.

Since proteins and perhaps other macromolecules are thought to be held in their native configurations by hydrogen bonds, their rupture may well be responsible for thermal death. Indeed, suggestive evidence indicates that hydrogen bonds are broken during the heat denaturation of proteins, and losses in biological activity are associated with this process. It is well known that some enzymes are active at higher temperatures than others either because all the bonds are stronger or because critical bonds are

stronger. Furthermore, some enzymes recover activity after boiling in water thus suggesting that any key bonds broken were reformed on cooling. Hence a relatively great heat resistance may be due either to stronger indispensable bonds or to reformation of the critical bonds on cooling. Since hydrogen bonds in general are weak it may be predicted that life can exist at temperatures above 100°C for only short periods of time, and that normally the limit will be considerably lower.

The observed temperature range for the survival of protoplasm, or as it has been called the *biokinetic temperature range*, probably extends from near absolute zero to about 150°C. Active growth and metabolism is further restricted to the narrower range within which water remains in the liquid state, and but few species are actually biologically active at the extremes of even this range. Of these species no single one can cover the entire interval.

Among the bacteria are found individual species which are adapted for growth at the lowest or at the highest temperatures at which active metabolism is possible, and like all other living forms each bacterial species and strain has a characteristic temperature growth range. While for the sake of convenience the species of bacteria have been catalogued into groups according to their temperature growth range, there are no sharp lines of demarcation between these groups, the temperature ranges of the different species probably constituting a complete spectrum of all the possibilities.

Based upon their growth temperature ranges bacteria may be subdivided into the following groups:

1) *Cryophiles* or *psychrophiles*, the species growing best below 20°C or at only slightly higher temperatures. The minimum temperature of growth is generally the lowest of all bacteria with growth of one species recorded at -7°C.

2) *Thermophiles*, the species growing best at temperatures between 55 and 60°C and some few species at still higher temperatures. The thermophilic organisms growing at the highest recorded temperatures are algae from natural hot springs for which growth has been claimed at 98°C.

The truly or obligately thermophilic bacteria are known as *stenothermophiles*, organisms growing at 60°C or higher temperatures and unable to grow much below 30°C. These organisms are distinguished from facultative thermophiles or *eurithermophiles* which while capable of growth at 60°C or higher values are able also to grow at temperatures lower than 30°C.

3) *Mesophiles*, those species with temperature optima intermediate to the psychrophilic and thermophilic bacteria. Mesophiles whose habitat is the intestinal tract or tissues of homoiothermic animals will usually grow at 40°C and some at even higher temperatures. The maximum temperature



for growth of most other mesophiles will usually be lower, many saprophytic species being unable to grow at 35 to 40°C and having a temperature optimum at about 20°C.

The range of temperatures within which an organism will grow and multiply is characterized by minimum and maximum limits and some intermediate most favorable or optimum temperature. These latter values are known as the *cardinal temperatures*. All other conditions being equal the car-

TABLE 26  
*Influence of the medium on the temperature limits of growth*

ORGANISM AND STRAIN	50°C					10°C				
	Lit-mus milk	Nitrate broth	Nutrient broth	Meat infusion agar	Nutrient agar	Lit-mus milk	Nitrate broth	Nutrient broth	Meat infusion agar	Nutrient agar
<i>Bacillus vulgatus</i> (Marburg Strain)										
C4.....	+	sl+	+	+	-	-	+	-	-	-
C5.....	+	+	+	+	+	-	+	-	-	-
C8.....	+	-	+	+	+	-	+	-	-	sl+
<i>Bacillus megaterium</i>										
C1.....	+	-	-	-	-	-	+	+	+	+
C10.....	+	-	sl+	sl+	+	-	+	+	-	+
<i>Bacillus mesentericus</i>										
C7.....	+	-	+	+	+	-	-	+	-	-
S2.....	+	-	+	+	+	-	sl+	sl+	-	-
<i>Bacillus albolactis</i>										
C9.....	+	-	-	-	-	-	sl+	+	sl+	+
17.....	+	-	-	-	-	-	-	sl+	-	-

dinal temperatures depend on the nature of the species or strain although the nature of the environment will cause these cardinal temperatures to vary for a particular species. In addition, the optimum temperature is prone to shift with time in response to changes in the physiological age of a culture. Even though the cardinal temperatures are not absolute the variations are sufficiently regular so that if conditions are specified and rigorously controlled the cardinal temperatures serve as useful aids in the taxonomic identification of bacteria. In Table 26 data illustrate the variation in growth at given temperatures with the nature of the nutrient medium.

## MINIMUM TEMPERATURE

It may be easy to comprehend why bacteria are inactivated at temperatures below which the water of their protoplasm does not exist in the liquid state, but the minimum temperature of growth of most bacteria is far above such a point. It is still more difficult to understand the very large differences in minimum growth temperature existing among bacteria. An attractive hypothesis supposes that the physical state of the protoplasmic colloids is the determining factor in establishing a minimum temperature. The chemical composition of species may vary sufficiently so as to account for differences in their biological zero points. The dispersion of cellular lipids and in particular the effect of lipids on the permeability of membranes would certainly depend on their physical state, a property dependent in turn on the temperature. In addition, the viscosity of protoplasm varies with temperature since the physical state of the lipids and proteins is temperature dependent.

Another hypothesis ascribes the minimum temperature to the accumulation of toxic products of metabolism which are unable to diffuse away or to be metabolically detoxified at rates rapid enough for their disposal. Unfortunately evidence is lacking which would permit an evaluation of these hypotheses as they apply to bacteria. In the case of a single stenothermophile the extracted lipids were found to be of rather constant composition independent of the temperature at which the organisms were grown. In addition the lipids were considerably more saturated than those from a mesophile. Thus the temperature of solidification of the lipids from the stenothermophile was rather high and close to the minimum temperature for growth. This case supports the hypothesis that the high minimum temperature of thermophiles may be related to the physical state of the intracellular lipids.

The generation time at a minimum temperature may be remarkably long, six or more hours for organisms with generation times as short as 15 minutes at the optimum temperature. For this reason it is desirable to incubate cultures for a week or longer when determining minimum temperatures of growth.

## OPTIMUM TEMPERATURE

For a given species it is meaningless to speak of the optimum temperature since there are numerous optima, each biological activity of the organism being characterized by its own optimum temperature (Table 27). The optimum temperature for a biological process is best defined as the inflection point on a temperature-velocity curve. The optimum temperature for the velocity of a biological process tends to shift downwards with time,

and changes of 10 to 15°C are not uncommon. The explanation for this phenomenon may involve a shift in the balance of enzyme synthesis and enzyme destruction. In other words, with the passage of time in a growing culture or resting cell suspension the rate of formation of new enzyme will decrease steadily or become negligible as limiting factors for synthesis become influential. As a result the optimum rate of activity will shift to lower temperatures where the rate of enzyme destruction becomes lower.

If this explanation is valid one might expect the optimum temperature to be fixed in steady states in which the rate of synthesis is not overtaken by the rate of deterioration of enzyme. Thus in the logarithmic phase of growth or under cultural conditions where the limiting factors for growth could be experimentally controlled by the removal of toxic metabolic end

TABLE 27

*Variations of optimum temperatures with the nature of the biological process being studied*

PROCESS	TEMPERATURE	
	<i>Streptococcus lactis</i>	<i>Streptococcus thermophilus</i>
	°C	°C
Multiplication rate. . . . .	34	37
Population density. . . . .	25-30	37
Fermentation rate. . . . .	40	47
Acid production. . . . .	30	37

(From Dorn & Rahn, 1939.)

products and the replenishment of the food supply, the shift of optimum temperature with time should be non-existent or greatly reduced. This hypothesis has not been verified in the laboratory since studies of temperature optima have been made with resting cell preparations or cultures in the stationary phase of population growth where steady states are not maintained and many factors become limiting.

MAXIMUM TEMPERATURE

The maximum temperature is the highest temperature at which a biological process is possible. On a temperature-velocity curve it would mark the end point of the portion of the curve of negative slope at which the velocity becomes zero. The change in sign of the slope of a temperature-velocity curve indicates a change to the predominance of destructive processes. As the temperature rises an increase in the velocity of reactions may be expected. However, the rate of change for all reactions will not be identical, the rate of enzyme deterioration becoming relatively greater at ele-



vated temperatures than rates of synthesis. In the language of physical chemistry this relationship may be reduced to the statement that the temperature coefficients of enzyme destruction or protein denaturation are greater than the temperature coefficients of new enzyme synthesis.

The fact that the maximum temperature varies with the chemical nature of the environment may be explained by assuming a disproportionately destructive effect of a rise in temperature upon individual mechanisms of synthesis. While data on this point are not numerous, in the case of luminous bacteria particularly good evidence exists for a variation in their nutritional requirements when tested on synthetic media at different temperatures. Thus it is possible to grow these organisms at elevated temperatures by supplying them with known nutrients which can be dispensed with for growth at lower temperatures.

A curious phenomenon repeatedly observed throughout the living world is the tendency for optimum temperatures to be closer to the maximum than to the minimum temperatures. In the case of the bacterial pathogens of warm-blooded animals the temperature of the natural habitat of the pathogen may be only a few degrees removed from the maximum temperature of growth. This probably is responsible in part for the observation that a temperature lower than the normal host body temperature may be best for the initial isolation and continued cultivation of the pathogen *in vitro*.

#### TEMPERATURE COEFFICIENTS AND BIOLOGICAL ACTIVITY

Within the biokinetic range temperature influences the rate of biological activity, and to the extent that a biological activity is based on temperature dependent physical and chemical processes this is a necessary result. It is therefore desirable to review some of the more general problems of temperature effects on physical and chemical processes.

A quantitative relation shows the influence of temperature on the rate of any given reaction.

$$\ln k = \frac{\Delta H_a}{RT} + C \quad (1)$$

where  $\Delta H_a$  is the heat of activation (see page 322),  $R$  the gas constant,  $T$  the absolute temperature,  $C$  a constant which has special significance in reaction rate theory, and  $k$  the reaction rate constant. This expression obeys the equation of a straight line obtained by plotting  $\log k$  against  $1/T$ . From such data it is possible to evaluate both the constant,  $C$ , and the heat of activation.

In many chemical reactions so far examined the rate constants at temperatures differing by  $10^\circ\text{C}$  themselves differ by factors of two to three

( $Q_{10} = 2$  to  $3$ ). This finding is conveniently expressed by means of a temperature coefficient:

$$Q_{\Delta t} = \frac{k_{t_2}}{k_{t_1}} \quad (2)$$

When  $\Delta t = 10^\circ\text{C}$ . the  $Q_{10}$  value noted above is the result. This coefficient for a  $10^\circ\text{C}$ . rise in temperature is usually about unity for photochemical reactions and radioactive decays indicating little effect of temperature on the rate of these processes. Physical changes yield values between one and two, closer to the former except in the change in viscosity of highly viscous systems or in processes based on surface forces such as the diffusion across an interface presenting a high energy barrier (see page 214). Radically different coefficients are observed for protein denaturation reactions where they reach 10,000 or greater for certain proteins in certain temperature ranges.

In view of the general categories into which temperature coefficients fall there is value in their determination for biological processes since clues may thus be gained of the nature of the key reactions in the process. Such studies are not conclusive because there is too much overlapping in temperature coefficients between types of processes and there is not yet a sufficient backlog of basic data to indicate the frequency of unusual temperature coefficients.

The heat of activation appearing in equation (1) is characteristic of the process and is sometimes known as the *temperature characteristic* and given the symbol  $\mu$ . Under ordinary conditions the heat of activation is equal to the *energy of activation*, an expression frequently encountered.  $\Delta H_a$  (or  $\mu$ ) is the number of calories required to activate a mole of the reactant to the energy state required for participation in the reaction concerned. Its magnitude may be determined from the slope of the plot of  $\log k$  against  $1/T$  or by calculation as follows:

Equation (1) is differentiated to yield

$$\frac{d \ln k}{dT} = \frac{\Delta H_a}{RT^2} \quad (3)$$

which is integrated between limits and solved for  $\Delta H_a$ :

$$\Delta H_a = \mu = \frac{2.3RT_1T_2 \log k_2/k_1}{(T_2 - T_1)} = \frac{2.3RT_1T_2 \log Q_{\Delta T}}{\Delta T} \quad (4)$$

Inasmuch as  $\Delta H_a$  for a reaction is essentially independent of temperature  $Q_{\Delta T}$  must diminish steadily as the temperature rises. Usually the change in  $Q_{10}$  of any reaction will be rather small because a  $10^\circ\text{C}$ . rise in tempera-

ture is only a 3 per cent increase in the absolute temperature at 27°C. (300°K.).

Quite frequently biological processes exhibit a considerable change in  $Q_{10}$  with temperature (Table 28). A great deal of attention has been devoted to explaining these variations. Three possibilities are worthy of consideration:

1) The change has no significance other than an expected change for  $Q_{10}$  with temperature for a process with a fixed  $\Delta H_a$  ( $\mu$ ). On this basis one would expect the variation in  $Q_{10}$  to be rather small. The student should note that the mathematical relation of  $Q_{10}$  to  $\Delta H_a$  ( $\mu$ ) makes for only a small change of  $Q_{10}$  with temperature over the limited extent of the biokinetic range and for the usual values of  $\Delta H_a$  ( $\mu$ ).

2) The temperature coefficient is influenced by the environmental condi-

TABLE 28  
*Change in  $Q_{10}$  of various biological processes with temperature*

INTERVAL	ACTIVITY OF LIVER LIPASE	FREQUENCY OF BEAT OF CAT'S HEART	PROTOPLASMIC STREAMING IN VALLISNERIA	YEAST ALCOHOLIC FERMENTATION
°C				
0-10	1.50	—	4.0	6.25
10-20	1.36	5.40	2.3	3.64
20-30	1.26	2.15	1.6	2.07
30-40	—	1.84	1.3	1.51
40-45	—	1.35	—	—

(From Belehradek, 1935.)

tions under which the biological process takes place when a change in temperature changes the internal or external environments. Since protoplasm is a multiphase or heterogeneous system the state of particular phases, the viscosity, pH, or oxidation-reduction potential may all be affected differently by a temperature change. Therefore, in a biological system temperature variations may well lead to uncontrollable and unknown changes in numerous variables. A change in temperature coefficient may be a reflection of this variation.

3) A new reaction has become the controlling or *master reaction* in the biological process at the temperature at which the  $Q_{10}$  changes sharply. Every biological process is based on one or more chains or consecutive series of either chemical or physical events. An orderly sequential arrangement of reactions is spoken of as a *catenary series*. Since each reaction in the chain would have its own characteristic temperature coefficient, a change in temperature would not cause an equal shift in the velocities of all the reactions in the series. Thus the *master* or slowest reaction would not neces-



sarily be the same at all temperatures. When temperature-velocity data are plotted, breaks in the slope of the curve presumably represent *critical temperatures* or the temperatures at which a different reaction in a catenary series has become the master reaction. The key reaction may be in the original consecutive series or in a branch of it.

A practical difficulty with this concept is that the changes observed in  $Q_{10}$  with temperature are actually often gradual rather than abrupt so that it is not possible to pinpoint the exact temperature at which a new reaction takes over as the master reaction of a series. In any case it is logical to try to exclude the two possibilities previously mentioned before accepting the shift in master reaction or critical temperature theory. Experimentally it may be impossible to eliminate the second of the above alternatives since the nature of the biological environment and the nature of the specific reactions in a catenary series are largely unknown. For these reasons the significance of the changes in  $Q_{10}$  with temperature has been a widely debated subject. The concept of changing master reactions has virtues but it has not always been applied critically. For this reason it is desirable to consider briefly some theoretical objections to its universal application.

#### THE MASTER REACTION OR CRITICAL TEMPERATURE CONCEPT

It does not seem possible to set up a theoretical sequence of monomolecular reactions, either reversible or irreversible, that will change from one rate controlling step to another over a small temperature range. Yet even in the relatively short physiological temperature range a number of normal processes show marked changes in values of the  $Q_{10}$ . Furthermore, the heat of activation  $\Delta H_a$  (temperature characteristic  $\mu$ ) changes for these processes, sometimes changing abruptly at the so-called critical temperatures (Table 29).

The fact that many biological processes are steady states makes hypotheses based on such simplified kinetic concepts quite unlikely. For in a consecutive series of reactions,  $A \rightarrow B \rightarrow C \rightarrow D$ , in a homogeneous phase and at a steady state it can be shown that changes only at the beginning of the series will have any appreciable, lasting effect on the rate of formation of final product. One may, therefore, conclude that since such a simplified model cannot explain the experimental data some other model must be sought. So far the search has been unsuccessful, and it becomes necessary to assume that the observed abrupt changes in heats of activation with temperature cannot be due to a change in the master or rate controlling reaction in any simplified sense.

Complexities that undoubtedly occur but cannot be evaluated at the present include the factors suggested on page 323 under point 2. These factors influencing the homogeneity of the system may thus profoundly

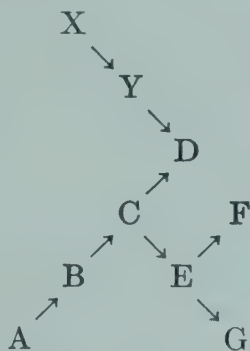
TABLE 29

*The dependence of  $Q_{10}$  upon temperature at various values of the heat of activation*

TEMP. RANGE °C	$\Delta H_a$ IN KILOCALORIES PER MOLE*				
	5	10	20	30	100
0-10	1.38	1.91	3.66	6.98	661
10-20	1.35	1.83	3.34	6.11	417
20-30	1.33	1.76	3.10	5.47	288
30-40	1.30	1.69	2.87	4.88	195
50-60	1.26	1.59	2.54	4.04	105
70-80	1.23	1.51	2.29	3.45	62
90-100	1.20	1.45	2.10	3.03	40
110-120	1.18	1.38	1.95	2.72	25.5

\* The first four columns cover the range of most chemical and enzymic reactions and the last column represents the more extreme protein denaturation processes. Observe that  $Q_{10}$  changes rather slowly with temperature except at  $\Delta H_a = 100,000$  cal/mole.

alter rate constants as the temperature changes. When one considers also the likelihood of parallel pathways to the same product or end-point, and the presence of innumerable branches in the metabolic system involving other starting or end products,



it is not surprising that overall heats of activation may vary. Ordinarily the variation might be progressive even under these conditions with the possible exception of changes of phase. Here abrupt transitions might occur since the velocity constants of heterogeneous reactions<sup>2</sup> can be extremely dependent upon the nature of the phases involved.

This discussion has pointed out the complex unknowns which enter into

<sup>2</sup> Heterogeneous reactions are those chemical processes for which the reactants occur in two or more separate phases. Therefore, the reaction must involve a transfer of material across an interface. The reaction of a liquid with a solid or with another immiscible liquid serves as an example.

any consideration of the master reaction concept and should emphasize that to a great extent due to these difficulties, much of the value of the idea of the master reaction is lost. If the term erroneously gives rise to a feeling that only series of consecutive homogeneous reactions are involved in metabolic processes, it should be discontinued. Although the utility of the master reaction concept thus becomes one of opinion, it cannot be understood to involve any clear theoretical impression of the mechanisms of the controlling steps of complex biological processes.

#### DEATH OF BACTERIA AT LOW TEMPERATURES

The death of bacteria at low temperatures probably is due to no one single cause. A very important consideration is the effect of low temperature on the colloidal state of protoplasm where any irreversible changes would be damaging to the cell. With objects as small as bacteria there are great difficulties in actually determining changes in the cellular colloids so that the idea as applied to bacteriology remains largely speculative.

The reduction of temperature under conditions favoring the crystallization of water is harmful by reason of disturbance of the colloidal state. The crystallization of water might result in the mechanical crushing and puncture of bacteria. This latter possibility is doubtful because of the repeated inability of investigators to find evidence in its favor. In addition there may be "salt death" resulting from the excessive concentration of crystalloids as the pure solvent separates from a solution. The processes of freezing and thawing are probably more destructive of bacterial life than either the actual temperatures themselves or the time of storage (see Table 30). The more rapidly the organisms can be cooled or thawed the greater the number of survivors.

If the cooling process is sufficiently rapid, water may be prevented from crystallizing from aqueous solutions. Rather water can assume an amorphous glass-like character, a state said to be *vitreous*. Rapid thawing of bacilli with their aqueous phase in a vitreous state would tend to prevent crystal formation as the temperature was raised toward the freezing point.

When bacteria survive the processes of freezing and thawing there seems to be little practical difficulty in maintaining cultures at below freezing temperatures for indefinite periods of time. Indeed bacteria have successfully withstood the temperature of liquid air although reports of survival at such low temperatures have been largely nonquantitative. Dehydration at below freezing temperatures (the process of *lyophilization*) is a popular and successful means for preserving cultures. Here too quantitative data are largely lacking (see Morton and Pulaski, 1938; and Hutton, Hilmoe, and Roberts, 1951).



## DEATH OF BACTERIA AT ELEVATED TEMPERATURES

Early in the history of bacteriology the problem of the death of bacteria by heating was studied. The bacteriologist, who usually must begin his work with sterilized materials, generally finds heat the most convenient sterilizing agency for his purposes. For successful sterilization the most heat resistant types must be destroyed, namely, bacterial endospores. The high thermal resistance of the endospore thus becomes a practical labora-

TABLE 30

*Death by continuous freezing and by alternate freezing and thawing*  
(Numbers indicate plate counts per ml)

CONTINUOUS FREEZING		ALTERNATE FREEZING	
<i>Eberthella typhosa</i>			
Inoculum.....	41,000	Inoculum.....	41,000
24 hrs.....	30,000	Frozen 3 times.....	90
3 days.....	1,800	Frozen 5 times.....	0
4 days.....	1,000	Frozen 6 times.....	0
5 days.....	2,500		
<i>Serratia marcescens</i>			
Inoculum.....	340,000	Inoculum.....	340,000
24 hrs.....	36,000	Refrozen once.....	2,600
30 hrs.....	42,000	Refrozen 2 times.....	280
48 hrs.....	14,000	Refrozen 3 times.....	15
96 hrs.....	4,900	Refrozen 4 times.....	0

(From Hilliard & Davis, 1918.)

tory problem as well as one of great scientific interest. Since endospores present the greatest obstacle to sterilization most of our bacteriological knowledge of death by heating is based on studies with endospores rather than vegetative forms. Fortunately the general problems to be solved and the variables affecting heating are not very dissimilar whether endospores or vegetative forms are under consideration.

It was not until about 1915 when the importance of and the simplified means for measuring hydrogen ion concentration penetrated into bacteriology that the true complexity of the problem of death by heating was realized. Environmental conditions prior, during, and after heating must be taken into consideration. Most scientific experimentation consists in fixing all of the possible variables but one and measuring the effects produced

by manipulating the single variable. The early investigators did not realize the broad significance of many factors (as the hydrogen ion concentration, concentration of organisms) or even know of their existence. Consequently in spite of its voluminous quantity much of the early work is open to criticism or is difficult to evaluate.

The following is a list of those factors that have been implicated in the heat resistance of bacteria:

1) Factors involved prior to heating.

a) Internal factors: heredity, chemical composition, properties of external surfaces, age.

b) External factors: nutrition, temperature of growth, pH, metabolic products, habitat.

2) Factors involved during heating: time, concentration of organisms, presence of clumps, nature of the suspending menstruum.

3) Factors involved after heating: conditions of growth, dormancy.

We can only know whether or not bacteria resist exposure for a given time and temperature by determining survival after heating. Strictly speaking, conditions after heating do not affect the thermal resistance of bacteria but they do determine our ability to recognize survival. This problem is a serious one in bacteriology where survival is determined by the indirect means of seeking gross signs (colony formation) of reproductive capacity after heating.

### *Factors Influential Before Heating*

HEREDITY. The literature is rich in conflicting reports of the heat resistance of the endospores of a given species. For example, *Clostridium botulinum* spores have been reported both to be destroyed within one hour at 80°C. and to resist heating for more than four hours at 100°C. Although the results in different papers are not strictly comparable because the conditions of growth and heat application are usually dissimilar the question to be answered is, how much are the reported variations due to hereditary factors instead of variations in environment? Attempts to increase heat resistance by processes of adaptation and selection are interesting in this connection but confusing, for the results of various authors do not agree. However, there is good reason to feel that adaptation to heat is generally unsuccessful. Once a pure line has been selected by single colony isolation, attempts at adaptation and further selection generally fail to result in strains of increased heat resistance.

The resistance of bacteria and their endospores to heating is the result of a complex of physiological and morphological characters. Insofar as these characters are subject to genetic law we may think of heat resistance as being limited by heredity. The differences of strains within a species

must be thought of as due to minor distinctions of physiology and morphology rather than to a single predominant genetic factor or gene controlling heat resistance.

**COMPOSITION OF THE ENDOSPORE.** Spores are antigenically different from homologous vegetative bacilli, nor do spores of different species possess any single antigen in common. These findings mean that the endospores of different species are characterized by differences in their protein and hapten chemistry. Studies of the antigenic constitution and spectroscopic evidence for a somewhat greater calcium content of spores have provided the only unquestionable indications of significant differences between the chemistry of spores and vegetative cells. These findings also suggest that in seeking the chemical basis for the differences in heat resistance among bacteria it will be more productive to think in terms of differences in the properties of individual organic constituents, particularly differences in the nature of individual proteins and lipids, rather than gross differences in the overall content of particular kinds of substances, such as water, nucleic acid, lipids, proteins.

**MEMBRANE.** There is a rough correlation of heat resistance with the nature of the spore coats as indicated by differences in mode of germination. The types most resistant to heat are those spores which germinate without an accompanying large increase in the volume of the spore prior to the rupture of the coat and in which the spore coat is persistent rather than readily lysed after germination.

**AGE.** Spores tested soon after sporulation and very old spores, particularly those left in contact with the growth medium, tend to be the least heat resistant. Not all authors are in agreement as to how important the age of the spore is in influencing heat resistance.

**NUTRITION.** The chemical composition of the medium in which the organism is grown influences the resistance of spores. The most complete study of this type involves *Clostridium botulinum* for which a direct relationship has been found between the nature of the fatty acids present in the growth medium and the resistance of the endospores. High molecular weight saturated fatty acids favor the production of spores of increased resistance.

**TEMPERATURE OF GROWTH.** The maximum resistance to heating occurs in endospores from cultures grown at their optimum temperature for growth and sporulation. In general the most favorable range for sporulation is narrower than and lies within that for growth. It may be defined as that temperature at which the greatest percentage of vegetative organisms form spores.

**pH.** The initial pH of media in which cultures are grown does not affect the resistance of spores as long as the medium is not so heavily buffered as



to prevent the shift of pH with growth to values at which sporulation occurs.

**METABOLIC PRODUCTS.** The long continued contact of spores with the culture media in which they have been formed is deleterious to their heat resistance. This effect is important in studying the relation of age of the spore to its resistance. Spores should not be permitted to age in culture media since we then deal with an added variable, namely, the effect of contact with metabolic products.

**HABITAT.** Contradictory results report both decreased and increased resistance of spores obtained from their natural environment as opposed to those from laboratory media. No differences have been noted for spores taken from liquid and solid media.

### *Factors Influential During Heating*

**TIME.** Without exception the effectiveness of killing by heat is a time-temperature relationship. The time required for sterilization decreases as the temperature increases hence it is ambiguous to use the expression *thermal deathpoint*. *Thermal death time* is an accurate expression of fact.

The  $Q_{10}$  for the heat death of endospores is generally high with values of from 3.8 to 10.7 reported, and with a rise in temperature the temperature coefficient increases. Vegetative cells show still higher  $Q_{10}$  values than spores reaching as high as 70. Dry spores and vegetative cells have a lower  $Q_{10}$  than when heated in the presence of water.

**CONCENTRATION OF ORGANISMS.** Vegetative cells and spores when exposed to heat do not all die at once, therefore, the greater the concentration of organisms the longer it will take to kill the last individual, that is, sterilize the culture. There are two possible explanations: that the thermal death curve is of an exponential nature and is indicative of a monomolecular order of death or that there is a normal variation in resistance of the individuals in the population. In the latter case the greater the number of organisms heated the further apart we might expect to observe the extremes of the variability curve. Presumably the greater the population density the more probable the inclusion of observable numbers of the most resistant individuals.

**PRESENCE OF CLUMPS.** Clumps of organisms have a higher resistance than individuals. This difference is explained on the basis that the organisms in the interior of the clump are subject to dry heat rather than moist heat. Actually no one has suggested how this hypothesis can be treated experimentally.

**NATURE OF THE SUSPENSION MEDIUM.** Spores suspended in nonaqueous media have a higher resistance to heat than do spores suspended in water. Placing mineral oil over cultures tends to increase resistance both by

trapping organisms at the water-oil interface and by increasing the opportunity for the formation of clumps. Heating bacteria in nontoxic oils is equivalent to sterilization in dry air.

With high sugar concentrations which tend to dehydrate the organisms the tolerance to heating is increased also. However, if sugar is heated in solution at the temperature of sterilizing autoclaves, particularly at alkaline pH values, toxic products are elaborated which combined with the heating produce a more rapid death rate. Finally, increases beyond certain concentrations of salts may reduce heat resistance while favoring it at low concentrations.

**pH OF THE SUSPENSION MEDIUM.** The heat resistance of spores is maximal in a pH range, usually slightly acid, and decreases with changes in pH beyond this range. The effects of acid pH values on heat resistance also depend on the nature of the acid used to lower the pH, oxalic acid having a particularly toxic action.

### *Factors Influential After Heating*

**CONDITIONS OF GROWTH.** Plate counts of heated vegetative organisms and of spores are higher in complex media than they are in media including only the barest minimum of nutrients for growth of unheated cultures. Spores that survive heating will germinate only if provided with the most favorable conditions for vegetative growth and may even be more demanding than vegetative organisms.

**DORMANCY.** Plate counts of heated organisms, particularly spores, will give increased counts as the length of the incubation period is increased. This dormancy is probably due both to the destruction of necessary enzymes which are re-elaborated slowly as well as to a greater sensitivity of injured organisms to any toxic materials which may be present in the medium.

### THEORIES OF DEATH CAUSED BY HEATING

Death produced by heating bacteria in air in the absence of water seems to be at least partially an oxidative process since desiccated organisms heated in an atmosphere of nitrogen or in a vacuum die at slower rates than in air. The nature of the reactions involved in the absence of oxygen are unknown. There is a surprising lack of quantitative data on the sterilization process by dry heat so that bacteriologists remain largely in ignorance of the mechanisms of death by dry heat sterilization. In air the death rate of spores of *Bacillus anthracis* rises sharply above 60°C. and then appears to level off at temperatures above 250°C. (fig. 57).

The chief theories of death from heat in the presence of water are the denaturation of proteins, asphyxiation, intoxication, and lipid liberation

theories. Heat denaturation of proteins including enzymes is an attractive explanation, particularly since the observed high temperature coefficients of death could thus be explained. The asphyxiation theory holds no attractions for bacteriologists. While it is true that increased temperatures will increase the demands for oxygen and simultaneously reduce the solubility of oxygen in an aqueous environment, asphyxiation cannot explain the death of anaerobes. At present there is no reason to postulate a different mechanism of death for anaerobes than aerobes.

When the temperature is raised the increased velocity of protoplasmic

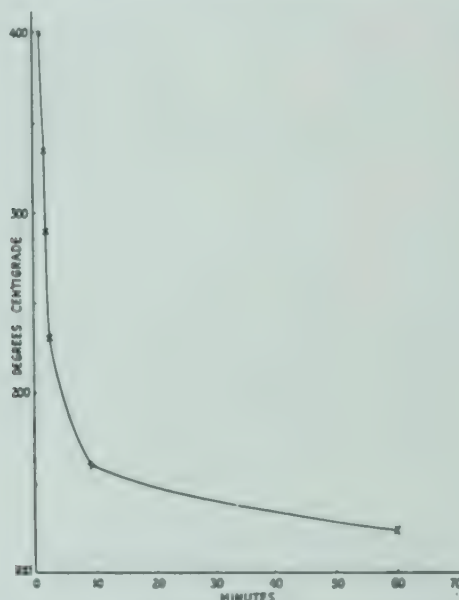


FIG. 57. Destruction of spores of *Bacillus anthracis* by dry heat at different temperatures.

(From Oag, 1940)

reactions may cause poisoning by an excessively rapid accumulation of metabolic end products. However, the rate of death at high temperatures is so extremely rapid that toxic products would seem to have little opportunity to collect in large enough quantities to cause the observed death rates. At temperatures slightly beyond the maximum growth temperature the intoxication theory may have merit. For endospores that succumb only at much higher temperatures the explanation has been sought elsewhere.

The lipid liberation theory explains heat death by changes in the physical state of the essential lipids in cells. In the general biological literature there is considerable indirect evidence for this point of view. Thus animals and plants which are killed at relatively low upper temperature limits contain fats of relatively low melting points. Fish possess fats fluid at room tempera-



ture and die at low temperatures. Mammals, most of whose fats are solid at room temperature, die at higher maximum temperatures than fish.

Cold blooded animal fats have a higher iodine number (unsaturation) and are thus more fluid than those of warm blooded animals. Oils from tropical seeds have higher melting points than those from temperate climates. Unfortunately, there is a paucity of this kind of work with bacteria. It is suggestive though that the heat resistance of spores of *Clostridium botulinum* is greater the higher the molecular weight of the fatty acids in the medium in which the cells are grown. In addition, preextraction of the sporulation medium by organic solvents results in spores of reduced heat resistance. Whether these findings can be correlated with an actual difference in the average molecular weight of the lipids present in spores grown on different media remains to be determined and would seem of great significance for evaluating the lipid liberation theory for bacteria.

The state of solution of lipids can affect the heat denaturation of proteins. Protein denaturation is enhanced in the presence of quantities of lipids just sufficient to saturate the aqueous phase. Thus observations with higher plants and animals that an increased content of cellular water increases the susceptibility to heat may be due not directly to a relationship between water content and the ease of protein denaturation but to the greater total quantity of lipids in solution and their enhancement of protein denaturation.

The protein denaturation theory and the concomitant concept of the destruction of essential enzymes has found the greatest number of adherents. In this regard the logarithmic order of death of heat killed bacteria has been offered as supporting evidence since the denaturation of proteins is of a monomolecular order. The theoretical significance of a monomolecular order of death for unicellular organisms like bacteria requires that death be due to the destruction of a single limiting molecule per cell, perhaps a lethal mutation. The lethal mutation theory does not have as sound a basis for explaining death by heating as it does for radiation inasmuch as the kinds of supporting data available are lacking for death by heat. For example, it is not clear that an increase in the number of cells exposed to heating is accompanied by an equivalent and theoretically predictable quantitative increase in the time it takes to sterilize a culture as would be demanded for a monomolecular order of death. The data available are few and not in agreement. If the normal variation in resistance of individuals of a biological population can explain the increased length of time it takes to kill all the individuals as the population increases, the per cent of survivors killed per succeeding unit of time would not be expected to remain constant. Eventually as the number of organisms is increased the prob-

ability of including the most resistant individuals would be very high, and beyond these concentrations an increase in the total population would have a smaller and finally negligible effect on the time required to sterilize the culture.

It is regrettable also that no thorough studies have been made of death rates of populations exposed intermittently to injurious temperatures. For if lethal mutation by heat is as valid a concept as lethal mutation by radiation, the heat resistance of survivors exposed to intermittent heating should not be less than the resistance of the original population. The total time required to sterilize should be no more nor less than in the case of heating the same concentration of organisms at the same temperature without interruption in the exposure period.

The high temperature coefficient of heat death favors the idea that a protein denaturation is the basic lethal reaction brought about by heating. In addition, the increasing knowledge of the wide differences existing among proteins in their capacity to resist heating and the increasing number of enzymes which have been found *in vitro* to be reversibly inactivated by heat makes possible the explanation of the many differences among organisms in their heat resistance on the basis of the tremendous variety in the nature of their proteins.

In this regard, in spite of contrary concepts of the nature of thermophily, the actual isolation from a stenothermophilic bacterium of enzymes, e.g., apyrase, malic dehydrogenase, succinoxidase, and cytochrome oxidase, of unusual heat resistance *in vitro* emphasizes the probable importance of proteins in determining the heat resisting characteristics of protoplasm. This hypothesis does not deny the role of other factors such as the nature and state of lipids, particularly as they may affect the heat stability of proteins.

#### HEAT AND THE ACTIVATION OF BACTERIAL ENDOSPORES

*Heat activation* is the increase in viability, increase in endogenous respiration, and decrease in germination time of endospores following their exposure to high temperatures for brief periods. Frequently even with other organisms than thermophiles growth in a culture is initiated only after a preliminary heat treatment of the inoculum.

As an example of the phenomenon the dehydrogenation of glucose in the presence of methylene blue by vegetative cells of *Bacillus subtilis* and homologous endospores may be cited. The vegetative bacilli dehydrogenate glucose at 40°C. much more rapidly than do the endospores. When both the vegetative organisms and endospores are heat shocked at 80°C. for 30 minutes and the temperature then reduced to 40°C. the vegetative cells are no longer capable of reducing methylene blue in the presence of glucose.

On the other hand the capacity of the endospore for dehydrogenation has been materially increased. A second heat treatment at 80°C. of the heat activated spores results in the destruction of enzymatic activity and reveals that the glucose dehydrogenase system of the heat activated endospores is more like that of vegetative cells than that of the original endospores. Heat shocking for different times at varying temperatures shows the degree of activation to be independent of time, and a function of absolute temperature only.

TABLE 31

*Effect of heating upon the activity of 1 milliunit of salivary apoerythein*

ONE HEAT TREATMENT*	APPARENT ACTIVITY OF 1 MILLIUNIT APOERYTHEIN
°C	
30	1.00
60	0.85
65	0.45
70	0
75	0.20
80	0.80
100	1.00
TWO HEAT TREATMENTS	
90 and 70	0.95
70 and 90	0

\* All heat treatments were made for five minutes. The order of exposure to the temperatures is as indicated for the samples heated at two different temperatures, the enzymatic activity being recorded on the material after heating at the second temperature.

(From Beerstecher and Edmonds, 1951.)

It is interesting to note that there exists a phenomenon of heat denaturation of certain proteins and enzyme systems which may be comparable to the heat activation of spores. A number of bacterial toxins, such as staphylococcal hemolysin and enterotoxin, a lecithinase of *Clostridium perfringens*, and more recently the apoerythein activity of human saliva, have been shown to be less sensitive to heat inactivation at higher than they are at lower temperatures (Table 31). In all of the described cases the materials tested have not been purified to a state of homogeneity so that it is not known whether an isolated protein or enzyme exhibits the same phenomenon. In the case of the lecithinase of *Clostridium perfringens* evidence is available to show that the anomalous thermal behavior may involve the reversible formation of heat stabile complexes, e.g., with divalent inorganic



cations. As yet no studies have been recorded which attempt to correlate any relation between these phenomena of anomalous heat stability of protein systems with heat activation of endospores.

### PRESSURE

Reports in the older literature indicate that bacteria can tolerate several thousand atmospheres of hydrostatic pressure, some differences existing in the behavior of different species. A sudden release of a high pressure was often found to be harmful, whereas a gradual reduction was innocuous. More recently pressure has been shown to vary in its effects and to be quite capable of modifying bacterial activity at considerably less than 1,000 atmospheres. In addition, the action of pressure cannot be considered apart from the influence of temperature. It has also been shown that a sudden release of pressure is not always as harmful as was reported in the older literature.

The response of bacteria to the mechanical application of hydrostatic pressure is related to the nature of their natural habitats (Table 32). In general terrestrial species will not grow at 600 times the normal atmospheric pressure and indeed may be killed at this and still lower pressures. Many marine forms, particularly those species living at or near the sea floor in a natural environment approximating 500 atmospheres pressure, grow readily under hydrostatic pressures of 600 atmospheres. Species isolated from the surface of the sea tend to be intermediate in their capacity to grow at increased pressures. The growth of some marine bacteria is actually favored by pressure, and the term *barophilic*, meaning pressure loving, is used to describe these species. Barophilic bacteria have been isolated from samples of the sea bottom at the greatest depths of the sea so far investigated 10,462 meters (34,324 feet). Many, but not all of these organisms grew well under a pressure of 1,000 atmospheres.

The effects of pressure on biological systems are manifold. Certainly pressure can modify the viscosity and elasticity of protoplasm, and such effects may be directly responsible for changes in the rates of metabolic processes altered by pressure. Fundamental data are available from the correlation of the nature of effects of pressure on metabolism with the temperature range for growth of the organism. The most comprehensive studies of these relationships have been made on the mechanism of light emission by luminous bacteria.

A reciprocal relation might be expected for the effect of hydrostatic pressure and temperature on bacterial growth and metabolism. This behavior would be an expression of Le Chatelier's theorem, namely, if a system is in equilibrium and an external force is applied, the system will shift in such a way as to minimize the effect of the applied force.

TABLE 32

Relation of the capacity of bacteria to grow at increased hydrostatic pressure to the temperature and natural habitat

CULTURE	300 ATMOSPHERES			400 ATMOSPHERES			600 ATMOSPHERES		
	20°C	30°C	40°C	20°C	30°C	40°C	20°C	30°C	40°C
<b>Terrestrial species</b>									
<i>Alkaligenes viscosus</i> .....	2+	3+	4+	2+	2+	2+	—	—	—
<i>Bacillus brevis</i> .....	—	3+	2+	—	+	+	—	—	—
<i>Bacillus megaterium</i> .....	—	4+	3+	—	+	2+	—	—	—
<i>Bacillus mesentericus</i> .....	—	4+	4+	—	3+	4+	—	—	4+
<i>Bacillus subtilis</i> .....	—	3+	4+	—	3+	4+	—	—	2+
<i>Clostridium bifermentans</i> .....	2+	4+	4+	—	—	3+	—	—	—
<i>Clostridium chauvei</i> .....	—	4+	4+	—	2+	3+	—	—	—
<i>Clostridium histolyticum</i> .....	—	4+	4+	—	—	3+	—	—	—
<i>Clostridium septicum</i> .....	—	+	2+	—	—	+	—	—	—
<i>Clostridium sporogenes</i> .....	—	4+	4+	—	2+	3+	—	—	—
<i>Clostridium welchii</i> .....	—	4+	4+	—	+	2+	—	—	—
<i>Escherichia coli</i> .....	2+	4+	4+	—	3+	4+	—	—	4+
<i>Mycobacterium phlei</i> .....	—	3+	4+	—	2+	+	—	—	—
<i>Mycobacterium smegmatis</i> .....	—	2+	2+	—	+	+	—	—	—
<i>Pseudomonas fluorescens</i> .....	2+	3+	4+	—	2+	4+	—	—	—
<i>Sarcina lutea</i> .....	2+	2+	4+	—	+	2+	—	—	—
<i>Staphylococcus albus</i> .....	2+	2+	4+	—	+	2+	—	—	—
<i>Staphylococcus aureus</i> .....	—	3+	4+	—	2+	3+	—	—	—
<i>Streptococcus lactis</i> .....	3+	4+	4+	+	4+	4+	—	—	4+
<b>Marine species</b>									
<i>Achromobacter fischeri</i> .....	4+	2+	*	2+	—	—	—	—	—
<i>Achromobacter harveyi</i> .....	4+	4+	—	+	4+	—	—	—	—
<i>Achromobacter thalassius</i> .....	—	4+	2+	—	—	3+	—	—	—
<i>Bacillus abyssus</i> .....	+	4+	4+	—	4+	4+	—	—	4+
<i>Bacillus borborokoites</i> .....	2+	4+	4+	—	4+	4+	—	—	4+
<i>Bacillus cirroflagellosus</i> .....	2+	3+	*	—	2+	—	—	—	—
<i>Bacillus submarinus</i> .....	2+	4+	4+	+	4+	4+	—	4+	4+
<i>Bacillus thalassokoites</i> .....	3+	4+	4+	2+	4+	4+	—	4+	4+
<i>Flavobacterium okeanokoites</i> .....	4+	4+	4+	4+	4+	4+	—	—	4+
<i>Flavobacterium uliginosum</i> .....	4+	4+	4+	4+	4+	4+	—	—	—
<i>Micrococcus infimus</i> .....	+	4+	*	—	+	—	—	—	—
<i>Photobacterium splendidum</i> .....	4+	4+	*	2+	3+	—	—	—	—
<i>Pseudomonas pleomorpha</i> .....	2+	4+	*	2+	3+	—	—	—	—
<i>Pseudomonas vadosa</i> .....	2+	4+	4+	2+	4+	4+	—	—	4+
<i>Pseudomonas zanthochrus</i> .....	4+	4+	*	4+	3+	+	+	+	—
<i>Vibrio hyphalus</i> .....	—	—	*	—	—	—	—	—	—
Mixed microflora from mud.....	4+	4+	4+	4+	4+	4+	4+	4+	4+

\* Failed to grow also at 40°C and 1 atmosphere pressure.  
(From ZoBell & Johnson, 1949.)





luminescence against the reciprocal of the absolute temperature reveals an inverted V shaped curve (fig. 58). The slope of the left portion of the curve is greater at atmospheric pressure than the right (low temperature) branch and yields a  $\mu$  value of approximately 54,000 in contrast to a  $\mu$  value of 17,000 for the right hand portion of the curve. The high temperature characteristic for the left branch is typical of a protein denaturation reaction. Therefore, a denaturation reaction involving the native luciferase, the known protein reactant of the system, has been introduced into the previ-

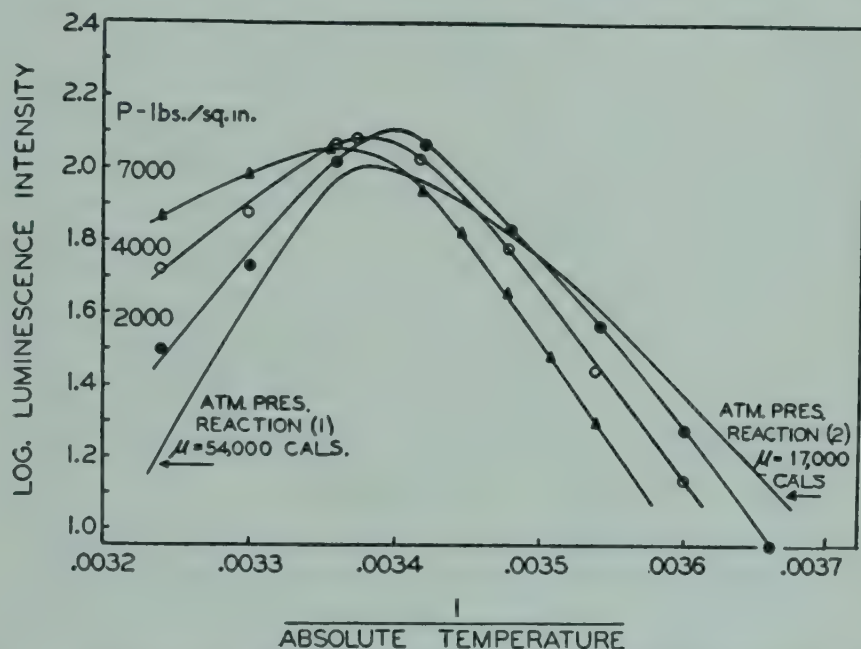
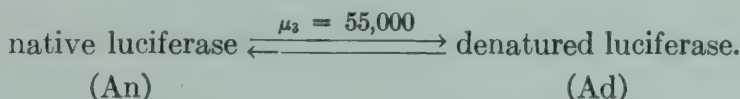


FIG. 58. The relation of the intensity of luminescence to temperature and pressure.  
(From Brown, Johnson, & Marsland, 1942)

ous outline of the luminescent system:



If pressure is applied to the luminescent system at temperatures above the optimum temperature, that is, on the left side of the intensity-reciprocal of absolute temperature curve, an increase in light intensity takes place. In other words, the inhibitory effect of temperature on luminescence is counteracted. In Figure 59 the data of such an experiment are plotted as a function of time. It will be noted that the slope of the curve for the system influenced by pressure parallels that of the control at atmospheric pressure and that both show a decrease in luminosity with time. Therefore, it is patent that whereas pressure is able to reverse the effect of temperatures

greater than the optimum temperature, there is still a consistent downhill trend of light intensity for both the control reaction and the one under pressure. These experiments can be conducted so that neither the concentration of oxygen nor substrate (luciferin) are limiting factors in which case the results are evidence for both an irreversible deterioration of enzyme

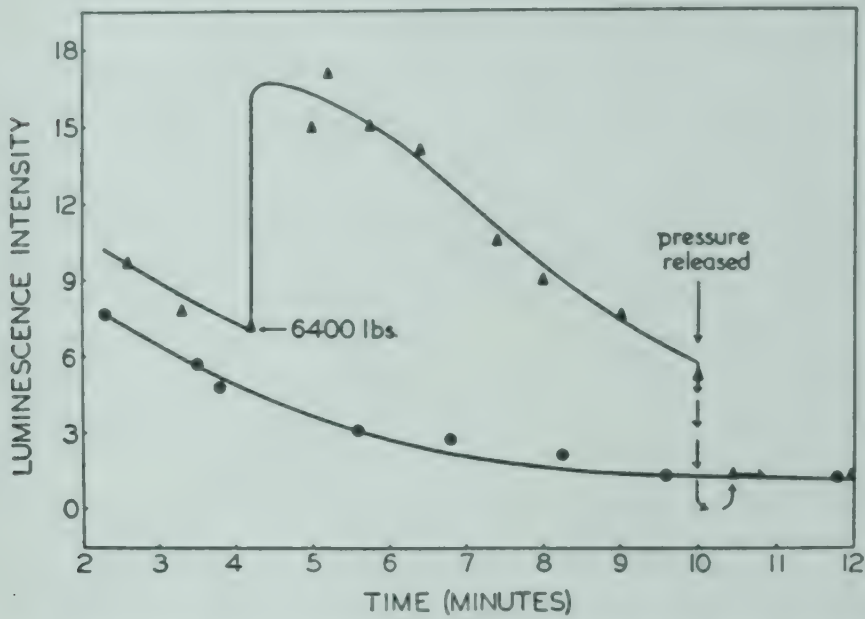
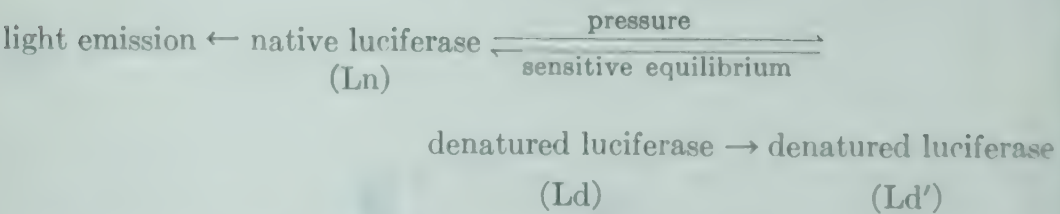


FIG. 59. The increase in luminescence under pressure at 35°C. with *Photobacterium phosphoreum*. This temperature is above the optimum for this organism.  
(From Brown, Johnson, & Marsland, 1942)

as well as a reversible reaction of which only the latter can be influenced by pressure, to wit:



What is the theoretical meaning of the described effect of pressure on the system? In the light of the Le Chatelier theorem and the ideal gas law, it is necessary to conclude that since the temperature is constant, an increase in pressure must be accompanied by a decrease in volume. Since increased pressure in the above case results in increased light emission it is logical to

conclude that the molecular volume of the native luciferase is less than the molecular volumes of the denatured states of the luciferase. This reasoning is of course consistent with other data on protein denaturation which indicate that denaturation involves a splitting of bonds and consequent unfolding and enlarging of the geometric configuration of the protein molecule. This picture is also consistent with the finding that at constant pressure a rise in the temperature decreases the intensity of luminescence. A temperature increase above the optimum would shift the equilibrium in the direction of the denatured metabolically inactive enzyme with its larger molecular volume since, according to the ideal gas law, at a fixed pressure the volume will vary directly with the temperature.

When the pressure is varied at a constant temperature below the optimum value the effect is to reduce the intensity of light emission, an action contrary to that already described at temperatures above the optimum. Results of a typical experiment over the entire range of temperatures for luciferase activity already have been given in Figure 58. From these findings it is obvious that temperature is critical in establishing the qualitative nature of the action of pressure.

Since the temperature characteristic for luminescence is different and much lower below the optimum temperature than above it and since the effects of pressure are also qualitatively different, it is possible to conclude that the optimal temperature represents a critical value. Below this critical temperature a different master reaction is controlling in the series of reactions leading to light emission than is controlling above the critical temperature. In the range above the optimal or critical temperature the master reaction involves a volume increase associated with an inactive state of the enzyme, but below the critical temperature the master reaction must involve a volume increase associated with an active enzyme state. Inasmuch as the activation of the luciferase molecule involves an excitation of an orbital electron or an increase in the mean free vibration path of the electron, this activation process may well be the step involving a volume increase and thus acts as the master reaction below the optimal temperature. In summation the master reactions of the luminescent system are;

above the optimum temperature  $An \rightarrow Ad$  with a volume increase

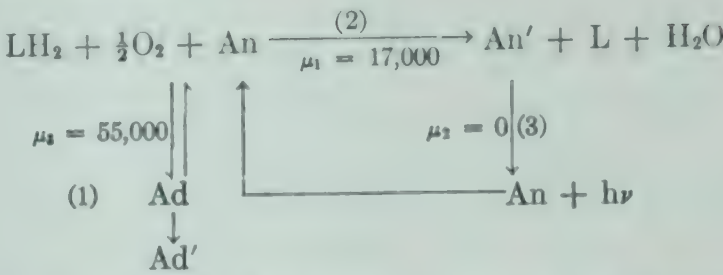
below the optimum temperature  $An \rightarrow An'$  with a volume increase.

Significant supporting data for this view are presented in Figure 60 which shows that with organisms having different optimal temperatures for luminescence the effects of pressure at a fixed temperature are qualitatively dissimilar.

It is now appropriate to summarize and schematically present by the following reactions the total theoretical picture of the temperature-pressure



effects on luminescence:



Temperature and pressure affect the above system in these ways:  
(a) At those temperatures below the optimum, reaction (2) is the limit-

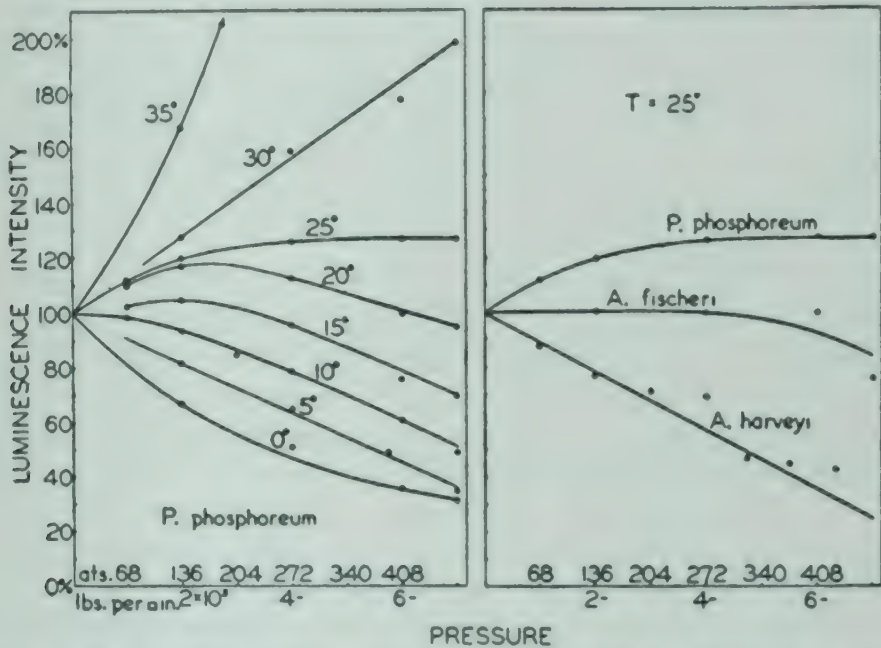


FIG. 60. Left diagram. Intensity of luminescence as a function of pressure at constant temperature.  
Right diagram. Relation of pressure effect on luminescence to the optimum temperature. *Photobacterium phosphoreum* has an optimum at less than 25°C., *Achromobacter fischeri* an optimum close to 25°C., and *Achromobacter harveyi* an optimum at less than 25°C.

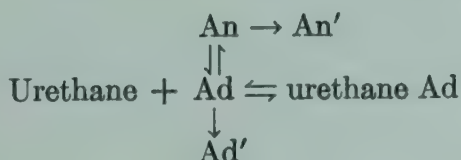
(From Brown, Johnson & Marsland, 1942)

ing or master reaction. Increased pressure reduces the velocity of reaction (2) and results in a decreased luminescence.  
(b) The optimum temperature represents a critical temperature, and pressure has little or no effect at this point.  
(c) At temperatures above the optimum, reaction (1) is the limiting or master reaction, for increased pressure shifts the equilibrium toward the active native state of luciferase resulting in an increase in luminosity.

Modern advances in the theory of the kinetics of chemical reactions, including the theory of absolute reaction rates associated with Eyring and his collaborators, permit the estimation of volume changes in equilibrium processes. An acceptable procedure involves the plotting of the logarithm of the rate of reaction against pressure for a constant temperature. A straight line should be obtained whose slope will be  $\frac{\Delta V}{RT}$ . Since  $R$  is the gas constant and  $T$  the absolute temperature at which the reaction is studied  $\Delta V$  can be calculated by substitution in the equation  $\Delta V = RT \cdot \text{slope}$ .

#### PRESSURE AND DISINFECTION

The developments in the theory of absolute reaction rates have provided a sound basis for the quantitative study and evaluation of the reciprocal effects of pressure and temperature on biological systems (Glasstone, Laidler, and Eyring, 1941). The experience with the luminescent system of bacteria has stimulated the application of similar types of analyses to other biological phenomena. In any biological system involving reversible changes in volume the same kind of reciprocal effects of pressure and temperature are expected as have been found with bacterial luminescence and can be interpreted in a quantitative manner. Thus the destruction of bacterial spores by heat must be considered to involve reactions accompanied by a volume increase since the application of pressure will reduce the death rate at lethal temperatures. Similarly any chemical disinfection reaction can be accelerated or inhibited by manipulating the pressure if disinfection involves a volume change in a reversible system. An example is the pressure reversal of the bacterial luminescence by urethane. In this case urethane is presumed to combine with a reversibly denatured state of luciferase:

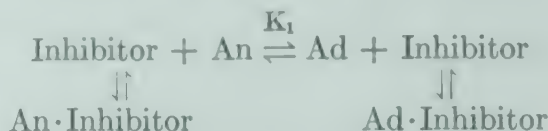


Thus in conformity with the Le Chatelier principle the application of pressure in the presence of urethane would shift the reaction in the direction of the smaller volume state of luciferase and tend to reverse the light dimming action of urethane poisoning. This effect would be particularly prominent at temperatures above the optimum for luminescence where the reversible denaturation reaction tends to be the rate controlling process. These theoretical predictions have been confirmed experimentally in studies of the effects of urethane on bacterial luminescence.

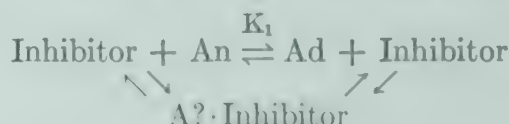
When an enzyme exists *in vivo* in the catalytically active state in a

reversible equilibrium with an inactive form and a volume difference exists between the two states, the study of the effects of a chemical inhibitor on the enzymatic activity may permit the correlation of inhibition with the particular state of the enzyme. There are two possibilities which have yielded to experimental manipulation and theoretical analysis:

Type I. The inhibitor combines in reversible reactions with both the active and inactive forms of the enzyme:



Type II. The inhibitor combines reversibly with only one of the states of the enzyme. Theoretical analysis is unable to distinguish the enzyme state with which the combination of the inhibitor takes place:



If the natural logarithm ( $\ln$ ) of the rate of uninhibited activity  $I_1$ , divided by the rate of the inhibited activity  $I_2$  minus one,  $\ln \left( \frac{I_1}{I_2} - 1 \right)$ , for an inhibitory concentration of the inhibitor, is plotted against the reciprocal of the absolute temperature a straight line will result in the case of a type I inhibitor. For a type II inhibitor a straight line will not result. However, a straight line will result for a type II inhibitor if the quantity  $\ln \left( \frac{I_1}{I_2} - 1 \right) \times \left( 1 + \frac{1}{K_1} \right)$  is plotted against the reciprocal of the absolute temperature.  $K_1$  is the equilibrium constant of the reversible equilibrium existing between the two dissimilar states of molecular volume of the enzyme.

For both the type I and type II inhibitors when  $\ln \left( \frac{I_1}{I_2} - 1 \right)$  is plotted against the molar concentrations of inhibitor a straight line is obtained. The slope of this line is the number of moles of inhibitor combining with each mole of enzyme (fig. 61).

In general, pressure effects on the activity of the inhibitor are related to the nature of the inhibition, either type I or II. An increase in pressure will reduce the inhibition by a type II inhibitor, whereas pressure will have no or little effect on a type I inhibitor (fig. 62).

The theoretical formulation for the experimental analysis for type I and II inhibitors is reviewed in the papers by Eyring and Magee, 1942, Johnson,



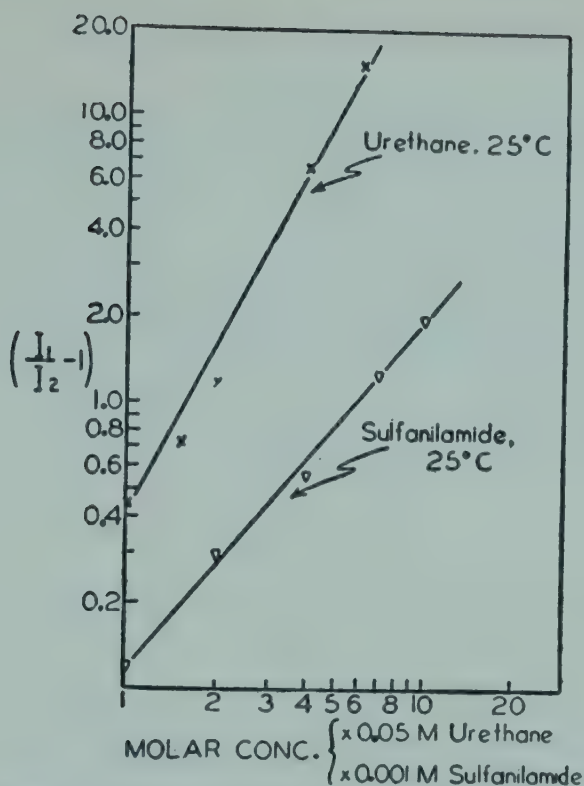


FIG. 61. The relation of the concentration of sulfanilamide and urethane to the amount of inhibition of the luminescence of *Photobacterium phosphoreum*. The slope of the urethane curve is 2, and that of sulfanilamide 1.2. The value of the slope represents the number of moles of inhibitor combining per mole of luciferase.

(From Johnson, Eyring, Steblay, Chaplin, Huber and Gherardi, 1945)

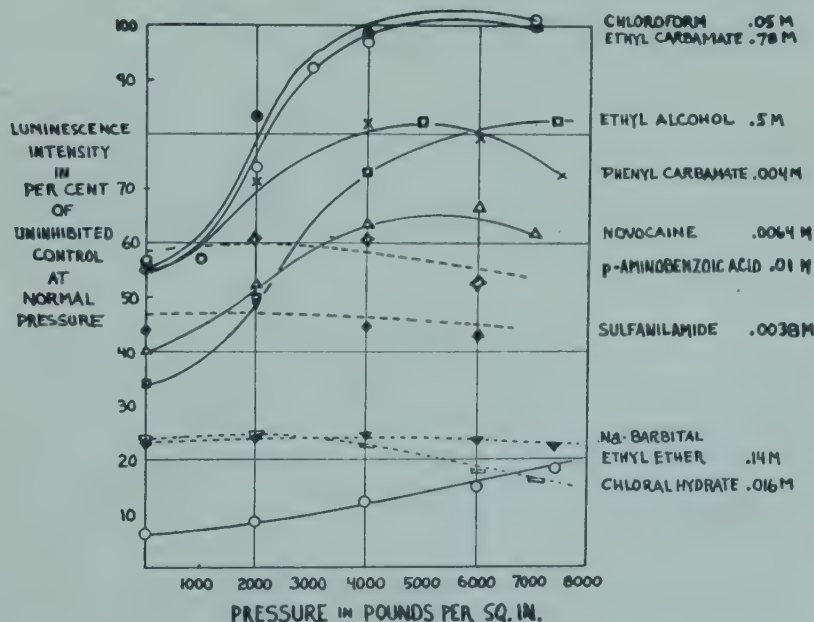


FIG. 62. The relation of the inhibition of luminescence by various drugs to pressure. The organism is *Photobacterium phosphoreum* and the temperature 17-18°C.

(From Johnson, Brown, and Marsland, 1942)

Brown and Marsland, 1942, Johnson, Eyring and Williams, 1942, and McElroy, 1943. An extension of this procedure to an analysis of the interaction (synergism and antagonism) of inhibitors in mixtures has been made by Johnson, Eyring and Kearns, 1943.

It should be emphasized that the foregoing analysis of temperature-pressure relationships in biological phenomena suggests the occurrence of changes in equilibrium systems involving differences in volume but does not actually provide proof that the changes are associated with a particular chemical reaction. Nor does it provide a choice as to whether the mechanism of the biological phenomenon involved is physical or chemical in nature.

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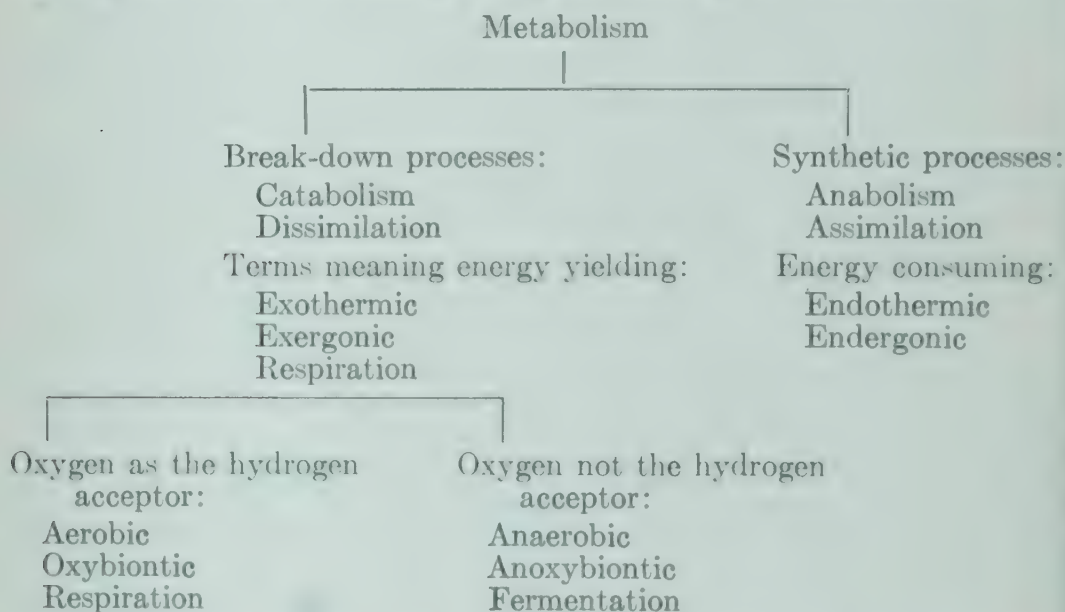
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## CHAPTER XI

# Nutrition of Bacteria

The nutrition of bacteria is an aspect of their *metabolism* which is the sum of the chemical processes continually going on in living organisms. These processes usually have been separated into two broad categories: the syntheses and the mechanisms providing energy, called respectively *anabolism* and *catabolism*. Nutrients are the external sources of raw materials utilized in either kind of metabolism, and a proper understanding of the food habits of organisms must involve an appreciation of the actual metabolic role of nutrients. For this reason it is necessary in a discussion of nutrition to employ the vocabulary developed to express metabolic phenomena. Since numerous synonymous terms are in current usage the following diagram may aid in distinguishing the meanings of these terms.



Note that there are two senses in which the term respiration has usage. The recent tendency among the students of intermediate metabolism has been to restrict the term to aerobic mechanisms of dissimilation, and it is this meaning which we shall employ.

As a group bacteria are the most omnivorous of organisms. They are capable of supporting their metabolic processes by the utilization of the

most diverse food sources. These sources may be completely inorganic in nature or include the most complex of organic molecules. Indeed there is probably not a single naturally occurring organic compound for which a persistent search would not reveal one or more kinds of bacteria capable of utilizing it as a nutrient. Most remarkable of all is the fact that bacteria exist which can utilize organic compounds having no known natural synthesis or existence outside the laboratory of the organic chemist. This ability is indeed a proof of the tremendous adaptability of bacteria, their capacity to respond successfully to a stimulus entirely foreign to their past history. If any further emphasis of this nutritional adaptability were required, one could present a catalogue of poisons to which individual types of bacteria are resistant and upon which they can feed. Included in the list would be such otherwise universally potent protoplasmic poisons as carbon monoxide and hydrocyanic acid. However, apparently a virtue may be overworked even by the bacteria since mutant types derived from wild populations have been described which are not only capable of utilizing particular poisons but are absolutely dependent upon these as food sources. Such are the streptomycin and sulfonamide dependent strains of acid-fast and other kinds of bacteria discovered by a number of investigators.

By the reason of their omnivorous habits the bacteria occupy an important niche in the cyclic biological utilization of the chemical elements. The total quantity of matter in the earth is fixed except for the addition of meteoritic material hence only the interconversion of this fixed quantity of matter can take place. Protoplasm is obviously active in determining the conversion of the biologically important elements at or near the earth's surface into particular molecular states. Since biological growth requires the net synthesis of complex molecules from simpler molecules, such growth must come to a halt when the supply of primary molecules is depleted. In fact biological growth has gone on over eons of time and probably will continue indefinitely until interrupted by an astronomic catastrophe because death and the bacterial activity associated with dead protoplasm result in a return of plant and animal constituents not directly utilizable by other plants and animals into the general biological economy. Bacteria can use as nutrients the products of higher forms, for example cellulose, woody tissues, and chitinous exoskeletons of insects. During the utilization of these complex products by bacteria the carbon, nitrogen, and other elements are by both direct and indirect means converted into states where they can be reused by green plants and animal species.

The great diversity of the habits of nutrition of the bacteria as a group is not reflected by the individual bacterial species. No one species is able to utilize all of the varied nutrients serving all of the bacteria. Apart from universally utilized nutrients such as water, carbon dioxide, phosphate, and



essential mineral salts, each bacterial species is characterized by a limited range of foods serving its needs. The nutritional requirements of wild strains of bacteria, even though subject to mutation, are sufficiently characteristic so as to have provided useful taxonomic criteria for bacteriology.

A modern nomenclature of the nutritional types of microorganisms applicable to bacteria which has gained favor is one published in the reports of the Cold Spring Harbor Symposia on Quantitative Biology (11: 302, 1946):

I. Nomenclature based upon energy sources.

A. Phototrophy

Energy chiefly provided by photochemical reaction

1. Photolithotrophy

Growth dependent upon exogenous inorganic H-donors.

2. Photoorganotrophy

Growth dependent upon exogenous organic H-donors.

B. Chemotrophy

Energy provided entirely by dark chemical reaction.

1. Chemolithotrophy

Growth dependent upon oxidation of exogenous inorganic substances.

2. Chemoorganotrophy

Growth dependent upon oxidation or fermentation of exogenous organic substances.

C. Paratroph

Energy apparently provided by the host cell. Implies parasitism.

1. Schizomycetotrophy

Growth only in bacterial cells.

2. Phytotrophy

Growth only in plant cells.

3. Zootrophy

Growth only in animal cells.

II. Nomenclature based upon ability to synthesize essential metabolites

A. Autotrophy

All essential organic metabolites are synthesized.

1. Autotrophy *sensu stricto*

Ability to reduce oxidized inorganic nutrients

2. Mesotrophy

Inability to reduce one or more oxidized inorganic nutrients—need for one or more reduced inorganic nutrients.

B. Heterotrophy

Not all essential metabolites are synthesized—need for exogenous

supply of one or more essential metabolites (growth factors or vitamins).

### C. Hypotrophy

The self-reproducing units (bacteriophages, viruses, genes, and so on) multiply by reorganization of complex structures of the host as well as by uptake of materials from the external environment.

Composite names are used for the concise characterization of a nutritional type with respect to the chief energy source as well as to the capacity for the synthesis of all essential cell constituents. An example is chemo-organoheterotrophic—*Escherichia coli*.

It is clear that foods may serve one of two purposes, namely, to be used as energy sources for the endothermic processes of synthesis or to provide the necessary structural components from which protoplasmic constituents are constructed. The building stone function may result in the incorporation of a food molecule as a unit into a structure or in its chemical breakdown and transformation into other primary molecules entering directly into synthetic processes. Thus, an amino acid used as a food may be assimilated, that is, incorporated as a unit in the synthesis of a protein molecule. On the other hand it may be deaminated, decarboxylated, or otherwise transformed chemically before some of its constituent atoms are incorporated into carbohydrate, lipid, nucleic acid, or protein and into several different molecules rather than a single molecule.

The foods which serve as energy sources may also serve as building stone materials. As a matter of fact catabolism or the energy yielding (*exergonic*) metabolism is not separate and distinct from the anabolic processes of energy utilization but rather can provide intermediate metabolites utilized in the latter. An analogy between biological growth and the construction of a building by human means would be a false one. In the human process the machines and human hands which provide the energy and control the direction of construction have an existence apart from the building materials and suffer no permanent physical incorporation into the structure. A true analogy would have to imagine the lumber, metal, and stone going into a building as being transmutable one into another and somehow expending a portion of their substance in providing the energy and direction for the final units which find their proper places in the constructed edifice.

Figure 63 is a schematic representation of the various foods used by bacteria and their general relationship to the protoplasmic structure. In the case of autotrophic organisms protoplasm is derived totally from inorganic sources. With heterotrophic organisms one or more carbon compounds other than carbon dioxide are required as nutrients.

With both autotrophic and heterotrophic organisms substances acting

as sources of energy are capable either of being directly phosphorylated or of yielding products capable of phosphorylation. Secondly, it is commonly accepted that substances acting as foods, no matter what their exact role, must be capable of penetrating the external boundaries of the organism. Bacteria convert insoluble and non-penetrating substances into soluble and penetrating compounds by the activity of extracellular enzymes or by contact with various enzymes in the outer envelope structures.

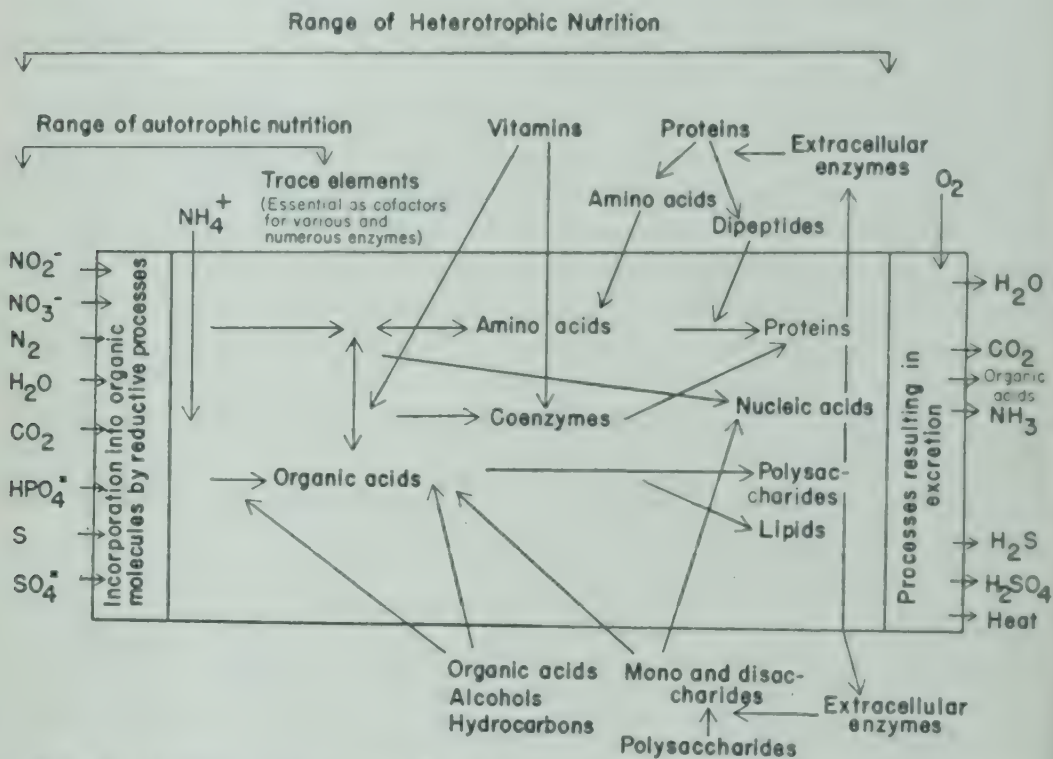


FIG. 63. A schematic representation of the nutrients utilized by bacteria, and their place in metabolism.

### THE UNIVERSALLY REQUIRED FOODS

There are a small number of nutrients which seem to be utilized by all bacteria as well as by other organisms which have been studied. These nutrients are water, phosphate, carbon dioxide, and certain mineral salts and might be labeled the universally required foodstuffs. In view of the similarity in the elements composing the living tissues of diverse organisms it might be anticipated that certain foods would serve all organisms. Actually the differences among bacteria in the foodstuffs required are not differences in the need for particular elements but are differences in the compounds of carbon, nitrogen, and sulfur which are assimilable. Furthermore, it is quite probable that the universally required foods were present and



served as nutrients during the original stages of the evolution of protoplasm. The need for them may be a primordial requirement which has never been lost and for which satisfactory alternates or, in the case of water and carbon dioxide, their adequate production from endogenous metabolic sources has not been accomplished by evolutionary processes.

If the heterotrophic hypothesis of the origin of life is accepted, the requirements of present-day heterotrophs for organic substances cannot represent a need for primordial foodstuffs. It has been pointed out in the previous discussion of this hypothesis that primordial organisms probably exhausted the original reserves of organic types of nutrients so that these no longer occur.

### *Water*

For growth and multiplication bacteria require high concentrations of water in their immediate environment. In this respect they are much more demanding than many other kinds of organisms, for example, the terrestrial fungi, hence it probably would be correct to consider all bacteria as aquatic organisms. In spite of their seemingly solid character and dry appearance, agar media and other solid foods used for the cultivation of bacteria require high concentrations of water as a part of their composition. Unfortunately, exact data on the water content which must be present in solid media do not seem to have been recorded.

While metabolism involves many chemical reactions which result in the production of water, it is doubtful that such metabolically derived water can satisfy to any large extent the water requirements for bacterial growth. Here again quantitative statements are not possible because there is a paucity of data upon which to base reliable estimates.

Water participates as a reactant in many metabolic reactions, and as a major constituent of protoplasm it is always present as a reactant in large excess. Thus many of the reactions involving water may mathematically simulate first order reactions though in truth they are not. Consequently, studies of metabolic reaction rates alone cannot be decisive in establishing the true order of metabolic reactions since the role of water as a reactant may not be evident from such data.

### *Mineral Salts*

In the natural environments of bacteria mineral salts are always present. That such salts are necessary for growth and multiplication is indicated by the absence of bacterial growth when synthetic media are used without them. A *synthetic* medium is one prepared from chemicals of known composition while a *non-synthetic* medium includes one or more ingredients of

unknown chemical composition, for example, yeast extract, peptone, beef extract, casein.

Since both the kinds and quantities of the salts which must be added to laboratory media vary with conditions, it is evident that the requirements for these materials are variable. In this connection it is known that mineral salts enhance, antagonize, or substitute for one another's biological effects. Different metallic ions can activate the same enzyme systems; the relative quantities needed, and ability of these ions to substitute for one another probably depending on their relative affinity for receptor sites on the apoenzymes. Since the ash content of bacteria tends to reflect the mineral content of media these organisms seem not to be particularly selective in their uptake of these substances. For these reasons it has not been possible to deduce unequivocally the mineral salts which are absolutely essential. If any are specifically required there is good reason to believe that iron and magnesium might be universally needed elements, requirements for which are completely satisfied from inorganic sources. While the evidence is not as convincing for potassium, manganese, calcium, and chloride, these ions may also be universally required foodstuffs. At the least these ions always seem to constitute a portion of both the natural environments of bacteria and bacterial substance. In fact their presence often favors more abundant growth than is obtained without their addition to media.

The participation of iron in biological oxidation-reduction reactions and of magnesium in the phosphorylation reactions indispensable for life processes could quite adequately account for a universal need for these elements in an inorganic state. While substitution for these elements in bacterial systems may frequently be demonstrated experimentally, such as manganese for magnesium, it is the naturally occurring case which is of most interest. The evidence would favor iron and magnesium as the naturally universally essential substances rather than substances which can be shown experimentally to substitute for them in isolated systems.

The experimental determination of an essential role for a nutritional substance involves observation of the effects following upon the exclusion of the substance from an otherwise complete medium. The difficulty with this approach to the problem of nutritional requirements in the case of mineral salts is the difficulty in excluding them from media, since the slightest contamination may suffice to satisfy the need of the organism. The order of magnitude of the growth requirements for inorganic salts is often  $10^{-6}$  molar or considerably less, such quantities being below sensitive chemical and spectroscopic methods for their detection. As a result, growth in the absence of the known addition of a mineral salt may not be taken as conclusive evidence of the absence of a mineral requirement since even



the use of highly refined chemicals and the purest water available may provide the necessary traces of ions. For example vacuum distilled iron employed as a spectroscopic standard contains less than 0.001 per cent cobalt as a contaminant. Yet the addition of traces of salts made from such iron is sufficient to satisfy the requirement for iron and also is sufficient to supply an excess of the cobalt required as cofactor in vitamin B<sub>12</sub> by organisms needing the vitamin. The satisfaction of this minute requirement is understandable since only about  $10^{-12}$  grams of cobalt per ml are required for bacterial growth.

Fortunately, the difficulty in studying mineral requirements by attempting to compound mineral-free media can now be overcome in certain instances by the use of chelating agents, a variety of which have become available since 1940. A *chelating agent* is a substance capable of combining with an atom (usually either hydrogen or a metal) by means of both ordinary valence bonds and coordinate bonds. Since both types of bonds are formed in a single molecule of the chelate compound, a ring structure results. The chelate compounds thus formed bind the atom and radically change its chemical properties. In fact when an effective chelating agent is employed a metal may be bound so tightly that the ordinary qualitative tests for the free ion are negative.

To determine a metal requirement the proper chelating agent can be added to a medium in order to create a deficiency of the metal in question. Thus employment of the chelating agent permits a quantitative estimate of the absolute mineral requirement, for it is only necessary to determine the minimum amounts of the element which must be added to varying concentrations of chelating agent in order to reverse the deficiency induced by the chelating agent. A plot of these quantities of a metal salt satisfying a nutrient requirement in the presence of a chelating agent against the concentrations of the chelating agent should yield a straight line whose intercept represents under the experimental circumstances the absolute quantitative requirement for the metallic ion. This method is very useful since it will quantitatively test the role of coordinating cations (those bound by chelating agent) in both synthetic and non-synthetic media under all sorts of conditions without the necessity for the prior removal of traces of the ion of interest which may be present and contaminating the reagents employed.

In practice it is often desirable to satisfy the mineral nutrition of bacteria without any preliminary investigation of exact requirements. For this purpose a large number of empirically devised salt solutions are used by investigators. On the premise that living organisms will contain all the essential minerals they need, organisms have been ashed and the product



added to media for the growth of other organisms. There are several difficulties with this approach. Ashing may result in the loss of volatile elements or yield insoluble oxides as products which make no effective contribution to a medium to which they are added. In addition, since bacteria are rather non-selective, their uptake of inorganic material depending largely on the nature of the medium in which they have been grown, the ash will contain non-essential as well as essential elements. More important, the ratios of the quantities of elements may not be optimal and may even be harmful since antagonism between ions is often observed at particular molar ratios of ions in mixtures. Toxic elements adsorbed from the original growth medium may also be inadvertently included in the ash.

### *Phosphorus*

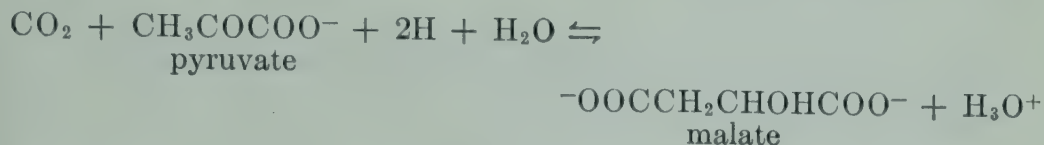
All bacteria require phosphorus which is obtained in the form of inorganic phosphate. While certain heterotrophic bacteria are able to derive their phosphorus from organic compounds they are not dependent on these sources and will utilize inorganic phosphate when supplied. The metabolic role of phosphorus is discussed in the chapter on metabolism.

### *Carbon Dioxide*

All bacteria so far studied require carbon dioxide for their growth although the requirement may be partially satisfied by carbon dioxide produced metabolically. Thus the amounts of carbon dioxide which must be drawn from an external reservoir of the gas can be considerably reduced. This fact has contributed to the historically difficult problem of detecting a carbon dioxide requirement for heterotrophic organisms by the conventional method of measuring the carbon dioxide taken up from a known volume of gas over a growing bacterial culture. However, this method was used by Wood and Workman in 1935 in demonstrating for the first time that heterotrophic organisms (propionic acid bacteria) can assimilate carbon dioxide. The use of the heavy isotope  $C^{13}$  in labeled carbon dioxide which can be detected in growing organisms by extremely sensitive mass spectrographic techniques or the use of radioactive isotopes have made possible definitive demonstrations that heterotrophic organisms assimilate carbon dioxide.

The assimilation of carbon dioxide into protoplasmic structure does not involve the building up of carbon chains by the direct formation of carbon to carbon linkages between consecutively reacting carbon dioxide molecules. Nor does it involve the direct reduction of carbon dioxide to formaldehyde or formic acid, a rather old hypothesis. In bacteria the fixation of carbon

dioxide involves the reversible formation of carboxyl groups as illustrated in the following instance:



In this reaction an enzyme is required and is accompanied by cofactors including the reducing agent merely indicated by H. The details of this and similar reactions are discussed in the chapter covering metabolism. Considering only the overall process shown, carbon dioxide disappears as the reaction proceeds from left to right. However, in so doing energy disappears (the reaction is endergonic) and must be made available by some other reaction system. (Mechanisms for the coupling of energy yielding reactions as sources of energy for endergonic processes are taken up later also.) Furthermore, if the assimilation of carbon dioxide serves any useful purpose the organic products must function as intermediates in cellular reactions, otherwise the compound formed (malate in this case) would merely accumulate as an end product.

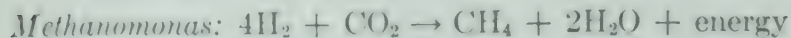
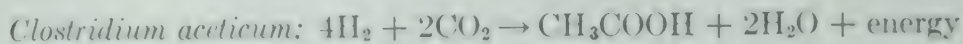
If an organism is permeable to the compounds resulting from carbon dioxide fixation, it should theoretically be possible to substitute these intermediates for the carbon dioxide. Similarly, any compound dissimilated to carbon dioxide should be capable of replacing the need for an external source of this gas. The latter of these expectations has been verified experimentally by the use of such substances as oxalacetate and succinate. It must be emphasized that the replacement of a need for an external source of carbon dioxide by addition of a compound to a medium does not necessarily signify that carbon dioxide is no longer required for growth. In fact there is good reason to believe that such a substitution satisfies the carbon dioxide requirement of an organism simply by increasing the supply of metabolically derived carbon dioxide.

Knowledge of the universal requirement for carbon dioxide should be taken advantage of in laboratory practice. The use of *capneic* incubators designed for the routine growth of bacterial cultures in atmospheres of controlled carbon dioxide content has been suggested. It is well known that the isolation of bacteria from their natural environments is often materially assisted by the use of increased carbon dioxide tensions although subsequent adaptation to laboratory conditions may reduce the need for such conditions after a series of transfers following isolation.

*Clostridium aceticum* and species of *Methanomonas* not only assimilate carbon dioxide but also use it as a hydrogen acceptor. These obligately



anaerobic bacteria obtain their energy from the oxidation of hydrogen but use carbon dioxide for this oxidation instead of some other hydrogen acceptor.



It has also been reported that carbon dioxide serves as a hydrogen acceptor in the metabolism of a typically heterotrophic organism, *Escherichia coli*.

### THE REQUIREMENT FOR OXYGEN

Some bacteria (*aerobes*) are capable of using molecular oxygen. The molecular oxygen does not become incorporated into protoplasmic substance. Instead the oxygen in the compounds of protoplasm is derived from water and other nutrients containing oxygen used by particular organisms. Molecular oxygen serves only as a hydrogen and electron acceptor and in the process is reduced to water. As such it participates at the end of the series of exergonic reactions serving the energy needs of aerobes.

Oxidation of foods involves not the addition of oxygen but rather the removal of electrons or electrons and protons. Thus the nutrients involved in exergonic metabolism may be viewed as donors which release electrons or hydrogen to acceptor substances. The transfer is not direct from a hydrogen donor to a hydrogen acceptor but requires the participation of enzymes, the *oxidases*, which possibly activate molecular oxygen and *dehydrogenases* which activate hydrogen donor substrates. The oxidation of nutrients may be summarized as shown in Figure 64.

Oxidases are able to reduce oxygen directly by transferring electrons and protons to the oxygen atoms with the resulting formation of either water or hydrogen peroxide depending upon the nature of the oxidase. Dehydrogenases ordinarily do not involve oxygen directly but act upon it indirectly through the mediation of an oxidase system. Various such processes are outlined in more detail in the chapter on metabolism since these systems are of fundamental importance in understanding metabolic processes.

It seems important here to comment on the general nature of the reduction of oxygen and various similar reduction reactions. Although it has been customary to speak of the transfer of hydrogen from a substrate to oxygen through the mediation of an oxidase, the reaction is actually not so direct. The overall process may be generalized as:





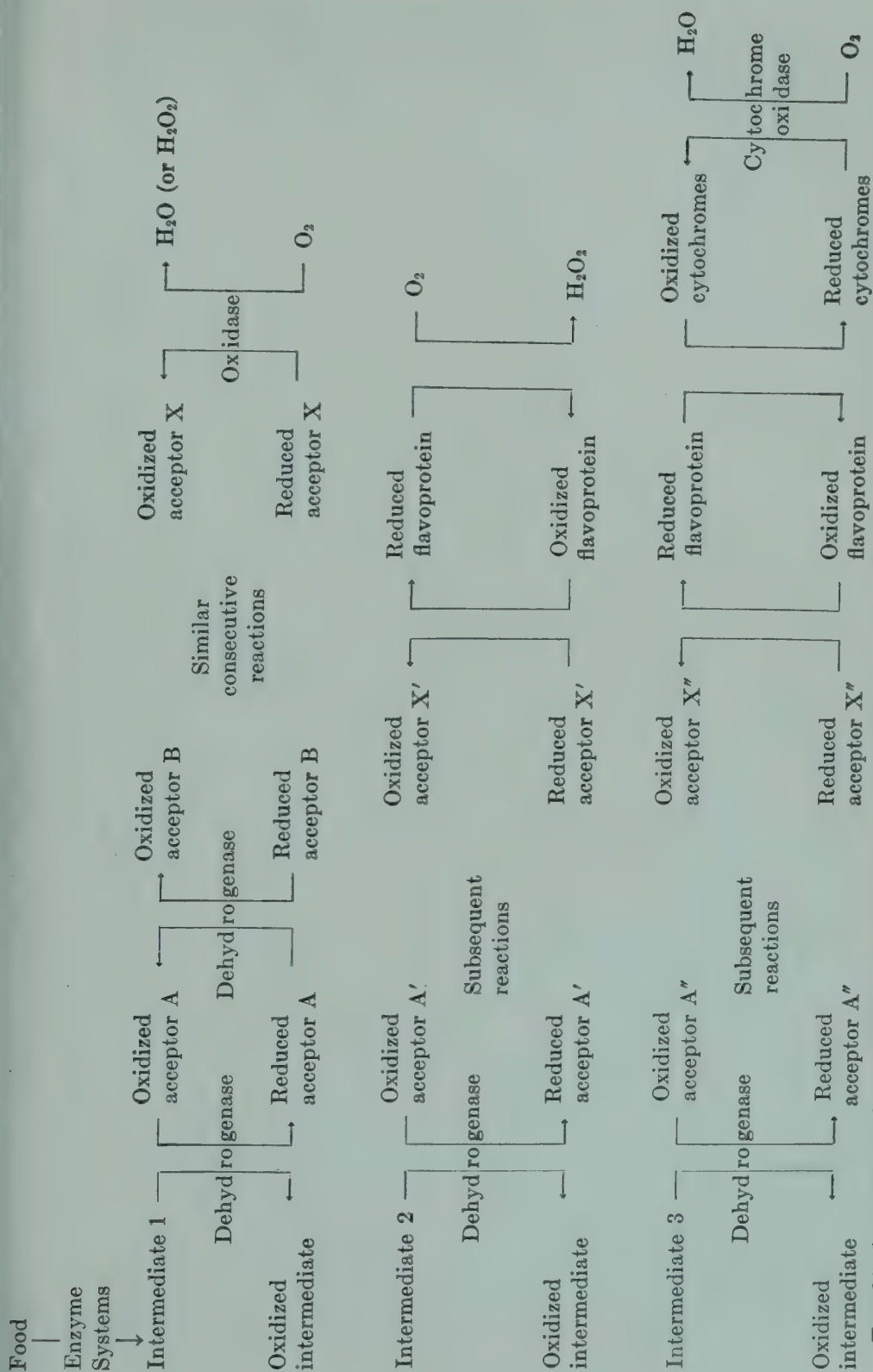
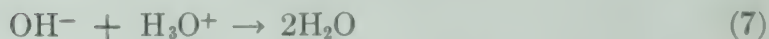
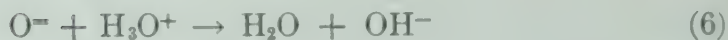
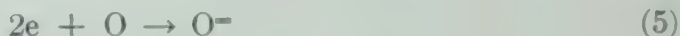
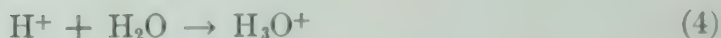
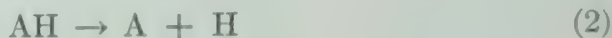
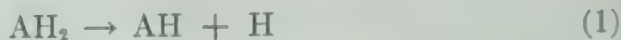


FIG. 64. A summary of the types of enzyme systems which have been described in the metabolic utilization of molecular oxygen as hydrogen acceptor.

where  $AH_2$  and  $A$  represent the reduced and oxidized substrate. However, some of the events occurring in this process must certainly involve electron and proton transfers. A hypothetical subdivision of the overall chemical reaction might be:



Reaction (3) presumably would follow very rapidly after (1) and (2) and would lead immediately to reactions (4) through (7). It would be difficult to demonstrate the independent occurrence of all of these steps or to prove that they are all correct in principle. However, some such scheme is indicated since studies with isotopes as tracers imply that the hydrogen atoms removed from a substrate are not necessarily the same individuals that are transferred to the oxygen atoms. This sort of mechanism does point out that both electrons and protons are transferred to oxygen and that for practical purposes one cannot separate the two processes. Therefore, when one speaks of the transfer of hydrogen one must realize that hydrogen atoms probably are not transferred directly but that both electrons and protons participate as intermediates. On the other hand oxidation-reduction reactions may occur without the involvement of hydrogen atoms or protons in certain cases. For example, in the cytochrome system electrons are transferred from the iron atom in one cytochrome molecule to the iron atom in the next succeeding cytochrome molecule and there is no need for the transfer of protons in such a system.

Apart from the biological utilization of oxygen as an electron acceptor, the presence of air can affect the capacity of nutrient media to support bacterial growth. Bacteria have been classified as *aerobes*, *anaerobes*, or *facultative* organisms depending upon whether they will grow in the presence of molecular oxygen, are inhibited, or indifferent to its presence.

Whenever activated hydrogen occurs there is always the possibility that hydrogen peroxide will be formed under aerobic conditions, and this product will invariably appear in the presence of certain oxidases. Thus organisms

sensitive to hydrogen peroxide will not grow aerobically unless they possess some enzymatic means for disposing of the hydrogen peroxide. In this role the enzyme catalase produced by many organisms converts hydrogen peroxide to water ( $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ ).

MacLeod and Gordon have divided bacteria into four groups:

- 1) Peroxide producers which also produce catalase. These include aerobes such as the aerobic spore formers, micrococci, diphtheroids.
- 2) Peroxide producers not producing catalase. Facultative organisms.
- 3) Non-peroxide producers not producing catalase. Facultative species.
- 4) Potential peroxide producers devoid of catalase. Clostridia are representative of this group.

While the logical suggestion has been made that the anaerobes are merely hydrogen peroxide sensitive organisms devoid of the capacity to produce

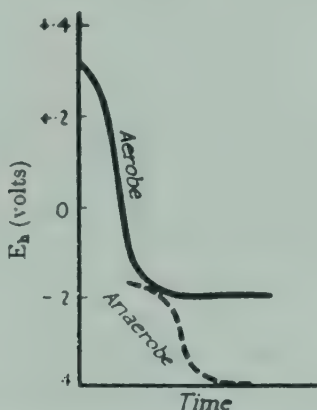


FIG. 65. Oxidation-reduction potential-time curve of a hypothetical aerobe and an anaerobe.

(From Hewitt, 1950)

catalase, and thus they are unable to grow in air, the absolute validity of this concept has been questioned.

The ability of an inoculum to grow out upon transfer to a fresh medium is dependent on the oxidation-reduction (O-R) potential of the medium, bacteria differing among themselves in the initial value at which they can grow. Once growth is under way the potential of the medium will drift to more negative values depending both on the nature of the organism and medium (fig. 65).

Since aeration of bacteriological media tends to give positive potentials and in view of the existence of a limiting upper value of O-R potential for initiation of growth, molecular oxygen would tend to be most harmful for organisms like the anaerobes which require the greatest reducing conditions



for the initiation of growth. In a study of this phenomenon it has been shown that at least some anaerobic species will grow even though aerated if various reducing agents are also present. Although such data indicate the importance of the oxidation-reduction potential, more than one possible effect may be involved. It may be assumed that critical enzymes are inactivated by the oxidation of key  $-SH$  groups. The aerobes are able to prevent such fatal damage; why aren't the anaerobes able to do the same? A common explanation has attributed all the damage directly to hydrogen peroxide by assuming that anaerobes are unable to decompose this toxic substance. Admittedly catalase is absent from many organisms, the clostridia for example. However, at least *Clostridium sporogenes* and *Clostridium saccharobutyricum* do have a mechanism for decomposing hydrogen peroxide even when small amounts are added externally. This unknown mechanism appears to function effectively by reduction of the peroxide until such time as so high a proportion of the reducing capacity of the cell is diverted to this process that the cell can no longer carry on the reductions normally needed for growth. Apparently reducing agents in the medium may be utilized for reduction of hydrogen peroxide thus sparing reducing substances synthesized by the cell and permitting growth in the presence of oxygen. If oxygen is absent no problem arises unless another strong oxidizing agent occurs in the medium. Thereupon a similar problem exists, and the anaerobe employs a part or all of its reducing power in dealing with the oxidizing agent.

It would seem that the metabolism of anaerobes normally functions to transfer electrons to acceptors of low potential. When an acceptor of high potential (good oxidizing agent) is present, the electrons are transferred so completely, and without yielding any available energy, that none are left for essential synthetic purposes.

No work has appeared in the literature on the corollary problem of the possibilities for the existence of lower limits of oxidation-reduction potential permitting growth. With anaerobic species this problem may be more apparent than real since these species can grow under quite strongly reducing conditions. In fact there is no evidence to suggest that such organisms cannot grow at potentials that are as low as can be attained in aqueous systems, the only systems of significance in bacteriology.

### AUTOTROPHIC BACTERIA

The autotrophic bacteria constitute a group of organisms which can derive carbon for anabolic metabolism solely from carbon dioxide. Obligate species, those which can only synthesize organic compounds from carbon dioxide, have been described as well as *facultative* organisms which are

capable of existence on carbon dioxide as the only carbon source in the absence of more complex organic nutrients. The energy required for the purposes of the syntheses associated with growth is obtained from the absorption of light in the case of *photosynthetic* species and from chemical reactions by the *chemosynthetic* species. While carbon monoxide and methane are used by a few autotrophic species it is still true that carbon dioxide is the only compound acting as the raw material for synthetic processes. The bacteria utilizing carbon monoxide obtain energy from the energy-yielding reaction:  $\text{CO} + \frac{1}{2}\text{O}_2 \rightarrow \text{CO}_2$ , the carbon dioxide produced in this reaction serving as the source of carbon for synthesis when an additional exogenous source of  $\text{CO}_2$  is not present. The organism oxidizing methane requires carbon dioxide for the synthesis of its protoplasm. The nutritional characteristics of autotrophic bacteria are summarized in Table 33.

The first pure cultures of autotrophic bacteria were isolated in 1890 by Winogradsky after his development of synthetic media entirely free of organic materials. As a solidifying agent he substituted silicic acid in place of the gelatin in common use in the early days of bacteriology, and to this day silica gel is the preferred means for the study of autotrophic bacteria in solid media. Apparently most of the naturally occurring organic products routinely employed in bacteriology contain toxic materials. Some of these substances which adversely affect the growth of autotrophic bacteria are high molecular weight fatty acids which also act upon heterotrophic species. This phenomenon has already been alluded to in the discussion of the phenomenon of dormancy. The growth of autotrophs in soil where organic matter is abundant in the face of their failure to grow in artificial media with organic constituents attests to the superior qualities of the natural environment. Apparently the physical structure of soils, the inclusion of both inorganic and organic colloids, and the presence of a mixed flora all act to reduce the effective concentration of toxic organic compounds by adsorption phenomena and possibly in other as yet unknown ways.

### *Photosynthetic Bacteria*

Among the autotrophic bacteria are pigmented species which can utilize radiant energy for anabolism (Table 33). The photosynthesizing pigment of the various species appears to be related to the chlorophyll *a* of green plants since it is a magnesium porphyrin differing from chlorophyll *a* only in possessing an acetyl group ( $\text{CH}_3\text{CO}-$ ) as a side chain rather than a vinyl group ( $\text{CH}_2=\text{CH}-$ ). The color of photosynthetic bacteria is the result of a mixture of the photosynthesizing green pigment and carotenoid pigments of unknown function.

The simplest overall equations representing the readily observable course



TABLE 33  
*Autotrophic bacteria*

*Photosynthetic types:*

- 1. Purple, sulfur bacteria (*Thiorhodaceae*, Molisch) anaerobic, develop in H<sub>2</sub>S media readily, and oxidize inorganic sulfur compounds to sulfate with reduction of CO<sub>2</sub>. H<sub>2</sub>S can be replaced by certain hydrogen donors, e.g., lower fatty acids, dibasic acids or molecular hydrogen. Growth factors not required.
- 2. Purple, non-sulfur bacteria (*Athiorhodaceae*, Molisch). Organic substances and molecular hydrogen serve as H<sub>2</sub>-donators; CO<sub>2</sub> reduced. Growth factors required. Certain species oxidize inorganic sulfur compounds.
- 3. Green bacteria (*Chlorobacteriaceae*, Geitler and Pascher). Occur in H<sub>2</sub>S media. CO<sub>2</sub> reduced, H<sub>2</sub>S being oxidized to free S which is usually deposited outside the organisms. Growth factors not required.

*Chemosynthetic types:*

NAME	N SOURCE	C SOURCE	ENERGY SOURCE		AUTOTROPHISM	O <sub>2</sub> -RELATIONSHIP
			Oxidation of	Reduction of		
<i>Nitrosomonas</i> , <i>Nitrosococcus</i>	NH <sub>4</sub> <sup>+</sup>	CO <sub>2</sub>	NH <sub>4</sub> <sup>+</sup>	CO <sub>2</sub> , O <sub>2</sub>	Obligate	Aerobic
<i>Nitrobacter</i>	NO <sub>2</sub> <sup>-</sup>	CO <sub>2</sub>	NO <sub>2</sub> <sup>-</sup>	CO <sub>2</sub> , O <sub>2</sub>	Obligate	Aerobic
<i>Beijerinckia</i> , <i>Thiothrix</i> , <i>Thiobacillus</i>	NH <sub>4</sub> <sup>+</sup>	CO <sub>2</sub>	H <sub>2</sub> S	CO <sub>2</sub> , O <sub>2</sub>	Obligate (Facultative?)	Aerobic facult. Aerobic Anaerobic Aerobic Aerobic
<i>Thiobacillus thioparvus</i>	NH <sub>4</sub> <sup>+</sup> , NO <sub>3</sub> <sup>-</sup>	CO <sub>2</sub>	H <sub>2</sub> S, S <sub>2</sub> O <sub>3</sub> <sup>-</sup> , S	CO <sub>2</sub> , O <sub>2</sub>	Obligate	
<i>Thiobacillus novellus</i>	NH <sub>4</sub> <sup>+</sup>	CO <sub>2</sub>	S <sub>2</sub> O <sub>3</sub> <sup>-</sup>	CO <sub>2</sub> , O <sub>2</sub>	Facultative	
<i>Thiobacillus thiooxidans</i>	NH <sub>4</sub> <sup>+</sup>	CO <sub>2</sub>	S, S <sub>2</sub> O <sub>3</sub> <sup>-</sup>	CO <sub>2</sub> , O <sub>2</sub>	Obligate	
<i>Thiobacillus denitrificans</i>	NH <sub>4</sub> <sup>+</sup>	CO <sub>2</sub>	S	CO <sub>2</sub> , NO <sub>3</sub> <sup>-</sup>	Obligate	
<i>Thiobacillus ferrooxidans</i>	NH <sub>4</sub> <sup>+</sup> , NO <sub>3</sub> <sup>-</sup>	CO <sub>2</sub>	Fe <sup>++</sup> , S <sub>2</sub> O <sub>3</sub> <sup>-</sup>	CO <sub>2</sub> , O <sub>2</sub>	Obligate	
<i>Hydrogenomonas</i>	NH <sub>4</sub> <sup>+</sup>	CO <sub>2</sub>	H <sub>2</sub>	CO <sub>2</sub> , O <sub>2</sub>	Facultative	Aerobic
<i>Carboxydomonas oligocarbo-</i>						
<i>phila</i> *	NH <sub>4</sub> <sup>+</sup>	CO	H <sub>2</sub> , CO	CO <sub>2</sub> , O <sub>2</sub>	Facultative	Aerobic
<i>Methanomonas methanica</i>	NH <sub>4</sub> <sup>+</sup>	CO <sub>2</sub>	CH <sub>4</sub>	CO <sub>2</sub> , O <sub>2</sub>	Facultative	
<i>Didymohelix</i> ,† <i>Sideromonas</i> , <i>Leptothrix</i> , <i>Crenothrix</i>	NH <sub>4</sub> <sup>+</sup>	CO <sub>2</sub>	Fe <sup>++</sup> , Mn <sup>++</sup>	CO <sub>2</sub>	Facultative, and obligate	

\* Has also been called *Actinomyces oligocarbo-*

† *Didymohelix* is now the genus *Gallionella* in Bergey's sixth edition.

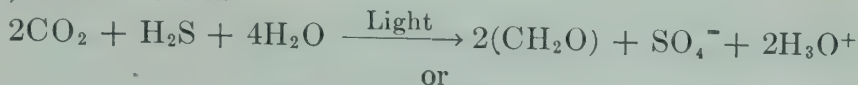
Adapted from Werkman and Wood, 1942)



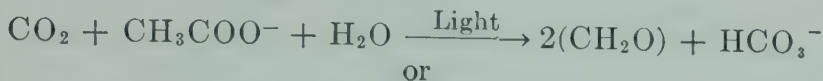
of bacterial photosyntheses are:



Purple, sulfur bacteria:



or



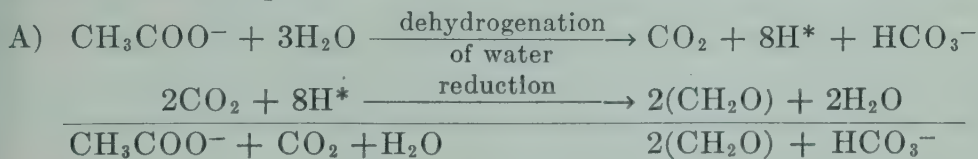
or



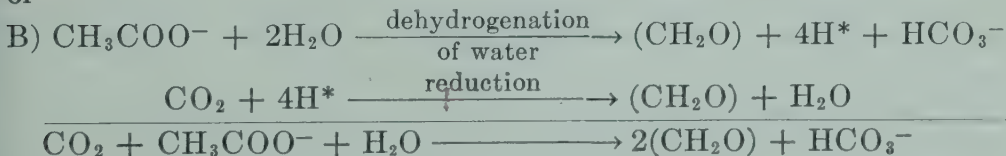
Purple, non-sulfur bacteria: Same as last two equations above.

These equations do not express the true course of the mechanism of photosynthesis but merely record the experimentally established data for the number of hydrogen donor molecules oxidized for each molecule of carbon dioxide fixed. Actually the hydrogen involved in the reductive assimilation comes from water. The formula  $(\text{CH}_2\text{O})$  is not meant to symbolize the true nature of the compound resulting from carbon dioxide fixation; it merely indicates that accompanying the fixation of the carbon dioxide molecule there is the synthesis of a more reduced compound than carbon dioxide. There is no reason to suspect that the nature of carbon dioxide fixation in photosynthesis in both green plants and bacteria is any different than it is for chemosynthetic autotrophs and heterotrophs. As such, photosynthesis is thought to involve the carboxylation of suitable intermediate metabolites. The reduction accompanying the photosynthetic process involves some other carbon atom than that of the carbon dioxide molecule fixed. Experimental evidence supports this view since the fixed carbon dioxide appears intracellularly first in the carboxyl groups of various organic acids.

In the case of species of *Thiorhodaceae* and *Athiorhodaceae* the participation of an organic hydrogen donor in photosynthesis may be visualized schematically as occurring in one of two possible ways. Acetic acid may be taken as the example:



or



(The asterisk denotes a free radical or atom.)

Note that the overall reactions are the same but that in scheme A both  $(\text{CH}_2\text{O})$  units, that is all of the assimilated product, are derived from carbon dioxide, while in scheme B, only half the total  $(\text{CH}_2\text{O})$  is derived from carbon dioxide and the other  $(\text{CH}_2\text{O})$  unit is formed directly from the acetate. If scheme B is true the organism is behaving like a typical heterotroph, since it is synthesizing organic compounds from a substance other than carbon dioxide as the raw material. In scheme A the acetate molecule acts as a source of raw material for anabolism only by first supplying a carbon dioxide molecule for assimilation. Which of these processes actually represents the true course of events is an unsettled problem, though evidence in favor of the direct assimilation of a part of the acetate has been obtained in the case of the purple bacteria.

If scheme A were the true one then the organisms using this method would not be typical heterotrophs in that they do not assimilate carbon in any form other than carbon dioxide, nor are they typical autotrophs since they are not metabolizing on an exclusively inorganic diet. A case of this sort points out the necessity of viewing the separation of nutritional habits into autotrophic and heterotrophic types as one of convenience which recognizes the existence of additional and intermediate possibilities, and not as a rigid natural division of dichotomous types.

Bacterial photosynthesis differs from the process in green plants in several ways: .

1. In bacteria chloroplasts are absent, the pigments being distributed throughout the cytoplasm of the organism.

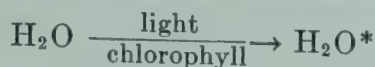
2. Bacterial photosynthesis occurs in the invisible infra-red region from 7500 to 9200 Å as well as in the visible spectrum. Differences in the absorption in the infra-red region by the various bacterial species are attributed to the existence of the photosynthesizing pigments in complexes with different proteins.

3. Molecular oxygen is not generated during bacterial photosynthesis.

4. Hydrogen donors varying with the nature of the species are necessary for bacterial photosynthesis. The donor substance may be hydrogen sulfide, an organic compound frequently an acid, or molecular hydrogen. This requirement accounts for the fact that photosynthetic bacteria occur in nature in brackish water and other places where the activity of other bacteria provides a source of utilizable hydrogen donors.

In spite of these differences the fundamental nature of the photosynthesis of green plants and bacteria is alike. A notable contribution to the subject of comparative biochemistry has been made by van Niel who has conclusively demonstrated the similarity in principle of the photosynthesis of different organisms. Photosynthesis involves the photochemical activation

of water (an asterisk is used to label an activated atom or molecule),



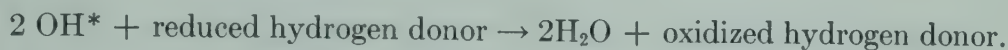
which is followed by the characteristic reduction accompanying photosynthesis



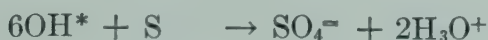
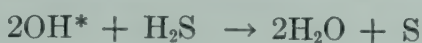
The differences between photosynthesis of the green plants and the bacteria then involve only the method for disposing of the hydroxyl groups from the activated water molecules. In green plants the hydroxyl groups appear as peroxides which in the presence of peroxidases or catalase break down to water and molecular oxygen



In the bacteria the hydrogen donors, other than water, participating in photosynthesis serve to reduce the hydroxyl groups derived photochemically from the activated water,



Specific examples of this generalized reaction would be,



Absorption of radiant energy at wave lengths at which photosynthesis goes on in green plants would result in bringing orbital or valence electrons of atoms to higher energy levels. Since bacterial photosynthesis utilizes radiant energy of longer wave lengths in the infra-red than do green plants, the activation in the bacterial system cannot raise the orbital electrons to as high energy levels as is the case with the green plants. As a result activated hydroxyl groups cannot react readily to form peroxide and so the bacteria need a different means for the disposal of these hydroxyl groups. The particular hydrogen donor system characteristic of the bacterial process, which of course must involve specific enzymes, provides the means. Figure 66 is a schematic presentation of photosynthesis.

This brief review of the nature of photosynthesis cannot close without emphasizing an important point, namely that the basic metabolism of the



photosynthetic organisms differs from that of chemosynthetic organisms only in the capacity to utilize a universally distributed and abundantly available hydrogen donor, water. The portion of Figure 66 labeled "dark" reaction is intended to call attention to the fact that only the activation of water in the photosynthetic process is photochemical in nature. The remainder of the process of utilization of carbon dioxide is not dependent on radiant energy and is identified as a series of temperature dependent "dark" reactions. Photosynthetic organisms deprived of light can still fix carbon dioxide, hence the historical origin of the term "dark" for labeling the non-photochemical reactions accompanying photosynthesis. The "dark"

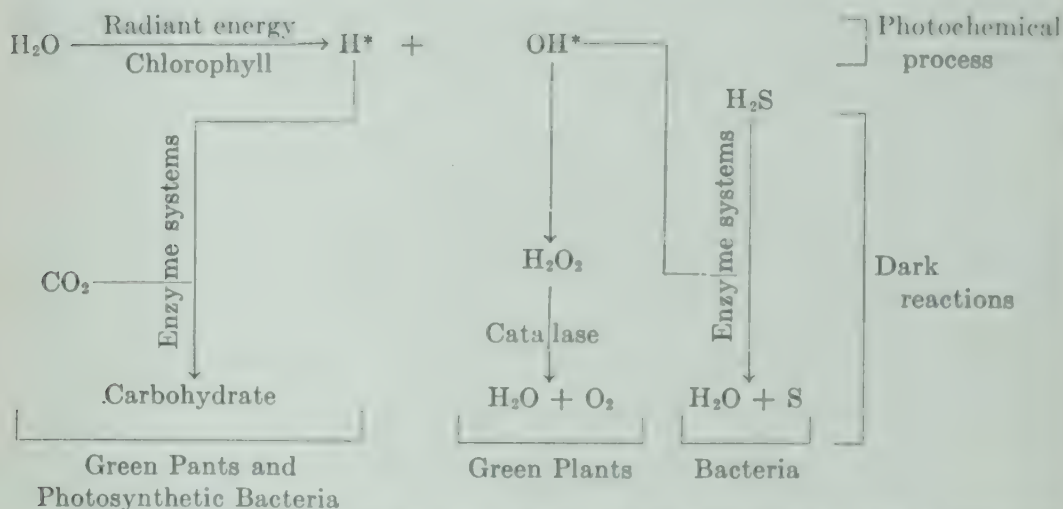


FIG. 66. Scheme of photosynthetic mechanism and associated "dark" reactions in green plants and bacteria.

(Adapted from van Niel, 1949)

reactions are probably similar in nature to those occurring in the fixation of carbon dioxide by chemosynthesizing organisms.

The fundamental unity in the nature of assimilation of carbon dioxide by photosynthetic and exclusively chemosynthetic mechanisms should not obscure the fact that photosynthesis is a more efficient process. Thus among those chemosynthetic mechanisms employed by organisms using molecular hydrogen in carbon dioxide fixation, four times the amount of hydrogen that is employed in photosynthesis is required for the chemosynthetic process. Also by employing water, photosynthesis is drawing upon a universally distributed and inexhaustible supply of hydrogen donor. If evolution is viewed as proceeding so as to increase the flow of energy through the organic world the greater efficiency of the photosynthetic process may indicate that it is the more recent arrival. In green plants photosynthesis utilizing only carbon dioxide, mineral salts, and water would seem to repre-

sent the acme of synthetic capacity. This capacity also requires a more complicated chemical constitution for the photosynthesizing organism which makes possible the more difficult synthesis of organic structure from the simplest inorganic components, a fact which reinforces the expressed view of the evolutionary order of appearance of photosynthesis.

In conclusion it should be recognized that the organic material of protoplasm is at a more reduced level than the empirical composition of the assumed product of photosynthesis,  $(\text{CH}_2\text{O})$ . This state of reduction is evident from the elementary composition of the quantitatively important components of protoplasm other than carbohydrate:

carbohydrate:  $\text{CH}_2\text{O}$

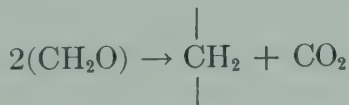
amino acids (average of 19 types):  $\text{C}_{5.6}\text{H}_{10.2}\text{O}_{2.5}\text{N}_{1.5}$

nucleic acids:  $\text{C}_{38}\text{H}_{49}\text{O}_{16}\text{N}_{15}(\text{PO}_3)_4$

short chain fats:  $\text{C}_{12}\text{H}_{20}\text{O}_6$

long chain fats:  $\text{C}_{54}\text{H}_{104}\text{O}_6$ .

Hence, in order to achieve a synthesis of such reduced states the product of photosynthesis must be in part oxidized in order to enable another part to be reduced. A generalized statement of this fact, which does not imply the exact mechanism of intermediary metabolism for achieving an increased state of reduction, would be:



or



The question of the actual metabolic pathways followed is considered in the chapter on metabolism.

### HETEROTROPHIC BACTERIA

Heterotrophic bacteria are those organisms unable to grow with carbon dioxide as the sole source of carbon. The nutrient requirements of members of this group vary greatly with the different species as do the functions performed by the organic foodstuffs. An organism may require an organic food as substrate for energy yielding oxidative metabolism, as a hydrogen acceptor or mediator in oxidation-reduction reactions, as raw material for synthesis, or as a coenzyme or precursor of a coenzyme. In addition some species require an organic source of nitrogen, and still others may also

require an organic source of sulfur for assimilation into protoplasmic structure. A single food may serve a number of these purposes simultaneously. Thus *Escherichia coli* utilizes glucose as a source both of energy and of carbon for synthesis; anaerobic spore-formers use amino acids as a source of energy as well as of carbon and nitrogen.

The autotrophs produce all of their organic constituents from carbon dioxide, and even the heterotrophs can fix carbon dioxide. The kinds of amino acids, coenzymes, and other organic compounds found in both types of bacteria are alike. Nevertheless, in spite of these similarities, the heterotrophs do require organic nutrients for synthesis. The reason for this need has not received a completely satisfactory answer. As the simplest raw material utilized, carbon dioxide must be incorporated through a series of intermediate steps into structurally more complex final compounds. Thus it might be expected that any organisms utilizing the simplest raw material would not require an exogenous source of organic compounds of intermediate complexity for synthesis into the final products. Another view, one based on the postulate of an evolution of autotrophism out of an original state of heterotrophism, supposes that such an evolution has involved the development of new means of carbon dioxide fixation. The difficulty with this answer resides in the lack of evidence for any differences in the fundamental mechanisms of carbon dioxide fixation by heterotrophic and autotrophic species. The whole course of investigations in comparative biochemistry has emphasized similarity rather than disparity. Possibly a wider group of organisms must be studied before differences will become evident. On the other hand the evolution of nutritional habits has proceeded with little change occurring in methods of carbon dioxide fixation by "dark" reactions.

The organic nutrients required for assimilation by heterotrophs compensate for the lack of a capacity to synthesize particular organic structures, an inability correlated with enzymatic deficiencies. Since the enzymatic constitution of organisms seems to be directly subject to genetic control the nutritional requirements of an organism imply either the absence of specific genes determining the production of particular enzymes, or that such genes if present exist in a form leading to an enzymatic deficiency. Thus the difference between autotrophs and heterotrophs need not be a matter of variation in the fundamental methods of carbon dioxide fixation but rather the existence of gaps in the metabolic pathways of heterotrophs beyond the first stages of synthesis associated with carbon dioxide. A basic necessity then for an understanding of the meaning of the differences between heterotrophic and autotrophic nutrition is the elucidation of the mechanism whereby genes affect enzyme synthesis.

Heterotrophic bacteria may mutate either to acquire a need for some



nutrient or to dispense with it. Since the number of genes within an organism is considered to be characteristic and constant the usual explanation for these nutritional mutations has been that the organism has suffered a change in state of particular genes rather than the alternate possibility of the absolute loss of genes or the *de novo* appearance of new ones. Yet in the evolution from heterotrophism to autotrophism there must actually have been numerous instances of increases in the total number of different genes in individual organisms. This increase must have occurred because autotrophs contain more kinds of enzymes than do the heterotrophs, equipping the former for their relatively increased means for syntheses. In such reasoning one assumes, of course, that there is a correlation between the numbers of different enzymes and genes present in an organism. If at any period in the past there were changes in the total numbers of different genes in organisms undergoing mutation to an increased synthetic capacity there is no *a priori* reason for denying the possibility to present day mutations. At the least the problems which are presented for solution concern the means for the recognition of a change in the number of different genes in asexually reproducing organisms and knowledge of whether the conditions necessary for such changes to occur have varied at different geological epochs.

While mutation phenomena have been much less studied with autotrophic than heterotrophic bacteria there is no doubt that nutritional variations occur in these organisms. From the nature of the case, autotrophs can only mutate to a state of synthetic deficiency, that is, an acquired need for an organic nutrient.

Wild populations of heterotrophic species of bacteria often constitute the strains with the greatest synthetic powers in spite of a liberal supply of nutrients in natural environments which could support the growth of the most nutritionally demanding strains. Yet the overgrowth of a parent population by a mutant with reduced synthetic capacity is sometimes observed in the laboratory. In these cases it is usually not clear how the nutritionally demanding types are better fitted for the laboratory than for the natural environment. Possibly, the rates of synthetic reactions involving the smaller molecular weight raw materials are slower or perhaps the deficient types may produce substances inhibitory for the non-deficient strains under conditions of laboratory culture. These suggestions have not been tested on any adequate scale.

### *Growth Factors*

*Growth factors* have been defined as those organic compounds required for growth which need be present in only minute quantities. In this sense a growth factor is synonymous with *vitamin*, the preferable term, since other

meanings have been attached to growth factor, namely, any substance required for growth, or any organic compound required for growth. It is this last definition we shall employ, reserving the term vitamin for organic compounds or growth factors required in only small quantities.

Growth factors may be *essential* in which case the bacteria must have them or no growth will take place. The observation that a substance is essential under one set of conditions does not imply a need for it under all other conditions of cultivation. *Staphylococcus aureus*, for example, requires uracil for growth under anaerobic conditions but will dispense with this requirement when grown under aerobic conditions with glucose present. Very often the dependence of the growth of an organism upon the presence of a compound will be eliminated under particular conditions because a precursor is being supplied. The further along a growth factor is located in a chain of synthetic reactions the more likely that substitute conditions and nutrients can be found which will supply a precursor.

Pyridoxal, pyridoxine, and pyridoxamine, various forms of vitamin B<sub>6</sub>, are all convertible to pyridoxalphosphate, the functional coenzyme in biological systems. As a result the addition of one of these forms of vitamin B<sub>6</sub> can eliminate the need for pyridoxalphosphate as a growth factor. Yet there are exceptions to this generalization. The yeast *Saccharomyces carlsbergensis* is actually more responsive to the unphosphorylated forms of B<sub>6</sub>. In this case the organism is probably least permeable to the pyridoxalphosphate and so does better with an exogenous supply of precursor material rather than with the compound resulting from the utilization of the precursor. A finding of this sort points to the complicating influence of permeability, and this difficulty reduces the chances of accurately predicting the nutritional preferences of an organism even when the relative positions occupied by growth factors in the stages of the complex of synthetic mechanisms are known.

As a rule growth factors are assumed to be functioning metabolites which contribute intermediates normal to the composition of organisms. Exceptions to this rule do occur. The best known is the case of mutant organisms having an absolute growth requirement for sulfonamides which certainly are not normal metabolites. The sulfa drugs ordinarily act as antagonists for *p*-aminobenzoic acid which is a naturally occurring metabolite. Apparently the organisms requiring the drugs, produce *p*-aminobenzoic acid in excessively large and harmful quantities thereby preventing their own growth unless the excess is neutralized by the presence of a sulfa drug in the medium. Among the B vitamins *p*-aminobenzoic acid is exceptional in that small excesses beyond the concentrations supporting maximal growth are demonstrably toxic. Thus this example also illustrates another impor-



tant fact, namely, that high concentrations of a growth factor may be more harmful than helpful.

In general the amount of growth in a bacterial culture is directly related to the quantity of growth factor present up to some maximum concentration of the nutrient beyond which no further increase in growth is observed. Whether the range of this plateau in the effect of concentration on total growth is small or large depends on the nature of the factor, the organism, and the conditions of growth. For most growth factors harmful concentrations have not been reported, but one wonders how much this has been due to a lack of interest in the subject rather than to any real absence of the phenomenon. In any case these considerations warn against any conclusion regarding the physiological usefulness of a compound based solely on data of toxicity.

The term *accessory* or *stimulatory* growth factor has been applied to those substances without which an organism can get along but with which it does better. The general function of such factors has been explained by postulating that they supplement the cellular stores of metabolites. Such supplementation would be effective if the rates of production of particular intermediate metabolites were too low to supply the quantities the organism is potentially capable of utilizing. Acetate is an intermediate metabolite resulting from the dissimilation of many substrates. It also serves as an intermediate in the synthesis of many other metabolites. If the supply of acetate from an endogenous source is small its addition to media helps achieve the maximal rate of growth potentially possible for an organism.

The vitamins required by various heterotrophic bacteria are in general the same ones needed by other organisms. The B, or water soluble, vitamins seem to play fundamental roles as coenzymes or coenzyme precursors in cellular metabolism. When an organism does not require these vitamins in a growth medium it does not necessarily signify that the organism does not use them but rather that the vitamin is synthesized. This has been proven by the direct chemical isolation of B vitamins from bacteria for which an exogenous source is not required (Table 34). A good example of this has been the determination of these vitamins in the autotrophic *Thiobacillus thiooxidans*. An alternate method involves the successful cultivation of vitamin-requiring bacteria in deficient media supplemented with extracts of or the killed organisms of non-vitamin requiring strains.

Are the B vitamins invariably required for metabolism, and are they invariably present in organisms either from external or endogenous sources? The general impression is that they are. Yet with bacteria it has become evident that folic acid and biotin may be exceptions, the case for biotin



TABLE 34  
*B vitamin content and synthesis by certain bacteria*

THIAMINE	RIBOFLAVIN		PANTOTHENIC ACID		NIACIN		BIOTIN		PYRIDOXINE		INO- SITOL	FOLIC ACID
Dry cells	Dry cells	Me- dium*	Dry cells content	Total syn- thesized dry cells	Dry cells content	Total syn- thesized dry cells	Dry cells content	Total syn- thesized dry cells	Dry cells content	Total syn- thesized dry cells	Dry cells content	Dry cells content
$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g, ml}$	$\mu\text{g, g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g, g}$	$\mu\text{g/g}$	$\mu\text{g/g}$
11-15		3.7	140	780	200	630	3.9	47.9	6.8	26.8	1400	2.8
			340	750	240	258	2.4	13.4	18	18.23	1600	1.0
			152-184				2.56-4.23					
	305-350				590							
33-96			93	318	250	1930	1.7	1.8	6.2	23.2	870	0.5
9.3			41									
12			100	100-130	250	330	3.4	24.1	6.8	16.4	1000	4.2
21			91	311	210	560	7.1	68.1	5.7	75.7	1700	1.8
26	2.9		120	172	240	470	4.1	34.1	11	34	1600	3.2
27												

\* Amount in medium after bacterial growth and exclusive of the riboflavin in the organisms.

† From Thompson, 1942.

‡ From Lee, and Burris, 1943.

§ From McIntire, Riker, and Peterson, 1941.

being a particularly strong one. Biotin has been implicated in the syntheses of aspartic acid and oleic acid. Certain strains of bacteria normally synthesize biotin, but when they are supplied with aspartic and oleic acids they show no detectable production of biotin. Thus the uses for biotin seem to be few and may be substituted for by external means. But the very intriguing question of how the enzymatic manufacture of biotin is stopped when the products resulting from biotin activity are supplied by an extra-cellular source is left unanswered. Here is a phenomenon which might be described as the reverse of the adaptive enzyme phenomenon.

In general bacteria do not require the fat soluble vitamins although an exception is Johne's bacillus (*Mycobacterium paratuberculosis*) which requires phthiocol, the aromatic portion of the vitamin K molecule. Many bacteria synthesize compounds of as yet unknown structure which have vitamin K-like activity in the clotting of blood. The function of such compounds in the bacteria is unknown. While some bacteria do synthesize carotenes there is no knowledge of what function, if any, these pigments related to vitamin A serve in bacterial metabolism.

Vitamin C is widely distributed in the plant kingdom and is required by many animals. It is not known to be needed nor to be synthesized by bacteria except for the exceptional report of its occurrence in *Serratia marcescens*. The addition of ascorbic acid to media may be useful to bacteria since this material may help poise the oxidation-reduction potential within favorable limits for growth. The role of vitamin C in this situation is not specific, and can be taken over by unrelated reversible reducing agents.

Among the *Eubacteriales* the genus *Clostridium* and the family *Lactobacteriaceae* are remarkable for the variety of species requiring great numbers of growth factors for *in vitro* cultivation. Various synthetic media for these organisms have been developed which include not less than 17 amino acids and as many as 9 vitamins. Even more deficient in synthetic capacity are a number of pathogenic bacteria, for example the leprosy bacillus, which have never been successfully cultivated through successive transfers on laboratory media. Whether this difficulty is due to requirements for a complex mixture of growth factors including the presence of one or many as yet unknown growth factors is a question which has not been answered.

Bacteria may be utilized as food by other organisms, and their food value in these cases is a reflection of their chemical composition. In nature some species of protozoa are completely dependent upon bacteria as their food supply. Curiously, the nutritive requirements of protozoa are not equally satisfied by different kinds of bacteria. Does this indicate that certain unknown growth factors are not universally distributed among bacteria? Or, since protozoa utilize the bacteria as solid foods, is the differing

nutritive value of bacteria only a reflection of limitations in the protozoal processes of converting solid foods to soluble and assimilable forms? In either case a difference in the structure of bacteria is indicated. The answers to these questions are incomplete in part because protozoologists have not been sufficiently precise in their taxonomic identification of bacterial strains and in part because bacteriologists have not much concerned themselves with bacteria as foods for other organisms.

More attention has been paid to the problem of utilization by animals of the vitamins which are produced by bacteria of the intestinal flora. There is no doubt that bacteria in the gut often make a valuable contribution to the nutrition of the host animal species by synthesizing vitamins. Much work of a definitive character remains to be done in this field.

**THE DETERMINATION OF REQUIREMENTS FOR GROWTH FACTORS.** The determination of requirements for growth factors in bacteria may be approached by either a chemical methodology in which one attempts to isolate and identify the growth factors present in a non-synthetic medium or by testing the capacity of empirically designed synthetic media for supporting growth. Historically the former method was the only one available, but since a long list of growth factors have been identified and as purified amino acids, purines, pyrimidines, and vitamins have become readily available commercially the latter method has taken precedence. In the case of organisms requiring unknown growth factors it is customary to supplement a basal synthetic medium composed of a minimum number of constituents with natural sources of the nutrient factor. In such a case the purification of the unknown growth factor is studied by noting the effects on growth of supplementation of the basal synthetic medium with isolated fractions of the natural source material.

When an organism will grow in a synthetic medium its growth requirements may be determined by withdrawing one growth factor at a time from the mixture. Thus the removal of an essential or stimulatory factor will be revealed by the reduced capacity of the medium to support growth. Those constituents which may be removed individually from the mixture without affecting growth are regarded as non-essential. Frequently a medium which is composed only of those substances whose absence from a mixture of growth factors adversely affects growth will not support growth. In such cases growth is only obtained when some or all of the so-called non-essential factors are added to the mixture of essential and stimulatory growth factors. The reasons for this anomalous situation are not always clear.

With the amino acids it has been shown, depending on the organism, that toxic effects may be exerted by an amino acid when added to a medium including only a few amino acids, although toxicity is not apparent in



mixtures containing a greater variety of amino acids. At the present time it is not possible to generalize as to the causes of these toxic effects and of antagonisms among the amino acids.

Some amino acids can act as precursors in the synthesis of other amino acids. Consequently in a medium containing a minimum number of amino acids a particular amino acid might serve and be required as a precursor material for amino acids missing from the medium. This role would be obviated when the missing amino acids were all included in a more complete medium. Thus one might explain the variations in the requirements for a particular amino acid arising from differences in the number of amino acids included in media. Similarly one could explain the absence of a requirement for a vitamin in a mixture of growth factors which included products resulting from assimilatory processes in which the vitamin normally participated as a coenzyme.

**PEPTIDES AS GROWTH FACTORS.** In common with all other organisms, heterotrophic bacteria synthesize intracellular proteolytic enzymes, and many are also capable of causing extracellular hydrolysis of proteins. It is characteristic of many proteolytic enzymes that they do not break proteins down to their individual amino acid components. Crystalline pepsin acting on egg albumin yields peptides composed of an average of seven amino acid residues. It is a frequent observation that hydrolysates of proteins produced enzymatically have a greater nutritive value for bacteria than do either acid hydrolyzed proteins which are supplemented with the amino acids destroyed by acid or completely synthetic media containing all the known amino acids. A low molecular weight peptide, *strepogenin*, has been described which acts as an accessory growth factor for some bacteria. Strepogenin can be replaced in media by the addition of a synthetic tripeptide, L-serylglycyl-L-glutamic acid. Tryptic digestion of a number of proteins results in products with activity like that of strepogenin. Logically all of the foregoing observations suggest the possibility that peptides might serve as more effective nutritional starting points for the synthesis of bacterial proteins than do mixtures of individual amino acids.

The most comprehensive investigations of the nutritive role of peptides have been conducted with synthetic peptides tested on mutants of *Escherichia coli* requiring amino acids as growth factors. As illustrated in Table 35 these studies have indicated that while a peptide may serve as a nutrient it is usually not more effective than the parent amino acids. Since more of the peptide is required in the study shown than of the individual parent amino acid for support of a similar amount of growth it is suggested that the peptide is split before it can serve as an amino acid source.

In the cases of the peptides of L-proline tested as growth factors with a mutant of *Escherichia coli* requiring proline, and of arginine peptides serving

in place of arginine in the nutrition of group D streptococci, growth promotion was more effective than when the individual amino acids were employed as nutrients. These results might imply the anabolic utilization of these peptides without prior hydrolysis to constituent amino acids. There are, however, two other possible explanations of these observations of specific growth promoting activity by peptides.

The permeability properties of bacteria might occasionally favor the

TABLE 35

*Effect of phenylalanine and tyrosine derivatives on the growth of mutant strains of Escherichia coli*

PHENYLALANINELESS STRAIN		TYROSINELESS STRAIN	
Compound	Micromoles per 10 ml for $\frac{1}{2}$ maximal growth in 24 hrs	Compound	Micromoles per 10 ml for $\frac{1}{2}$ maximal growth in 24 hrs
L-phenylalanine.....	0.1	L-tyrosine.....	0.09
L-phenylalanine.....	0.2	Glycyl-L-tyrosine.....	0.13
Glycyl-L-phenylalanine.....	0.1	L-glutamyl-L-tyrosine.....	0.19
L-glutamyl-L-phenylalanine...	0.3	L-tyrosylglycine.....	0.13
L-phenylalanylglycine.....	0.15	L-tyrosinamide.....	3.1
L-phenylalaninamide.....	3.3	Glycyl-L-tyrosinamide.....	64
Glycyl-L-phenylalaninamide.....	95	Carbobenzoxylglycyl-L-tyro-	
Carbobenzoxylglycyl-L-phenylalanine.....	401	sine.....	235

The fact that twice as much DL-phenylalanine is required as L-phenylalanine for support of the same amount of growth indicates that only L-phenylalanine serves as a growth factor, the D-phenylalanine is not utilizable nor toxic in this case.

Since much more of the compounds with the carbobenzoxy group substituted on the amino group and of amide group substituted for the carboxyl group are required for growth than the parent type amino acid, these derivatives are not possible intermediates in the synthesis of protein.

(From Fruton and Simmonds, 1949.)

intracellular penetration of peptides. Available evidence indicates that glutamic acid may be transported across the membranes of gram positive bacteria as a peptide. In addition amino acids are subject to enzymatic attack and degradation. Thus a portion of the supply of an amino acid in a medium might be lost to synthetic metabolism because of deamination, decarboxylation, or other degradative change. A peptide could be more resistant to such attack. Hydrolysis of the peptide to free amino acids limited in time to the period immediately preceding the entry of the peptide into anabolic processes might thus act to spare more of the supply of amino acids for anabolism than in the situation in which the amino



acids are supplied directly as growth factors. This is the suggested explanation for the enhanced nutritive value of peptides of arginine for group D streptococci in spite of the possession by the organisms of an active arginine dihydrolase.

When for purposes of growth a peptide serves as well as but not better than its individual amino acids it is probable that the rate of hydrolysis of the peptide to its constituent amino acids is not limiting in the utilization of the peptide by the organism. On the other hand, if cultures with the amino acids produce more rapid growth than cultures with the peptide, then the rate of hydrolysis of the peptide probably is limiting for growth in the presence of the peptide.

### JUDGING THE VALUE OF A MEDIUM

One often wishes to compare the effectiveness of different media for the growth of a particular organism. Necessarily any valid judgment must be based on some measurement of the amount of growth supported by the media. Thus one may resort to such procedures as plate counts, determination of bacterial nitrogen, and in the case of liquid cultures optical density measurements. The yield of growth per mole of food factor consumed is an obvious unit for comparison.

With complex and non-synthetic media it may be tedious and difficult, if not experimentally impossible, to relate the amount of protoplasm synthesized to the actual amount of various nutrients consumed. Then again, it may be desirable for practical reasons to know how various empirically compounded media compare without regard to the actual synthetic efficiency of organisms on those media. In such cases one might compare yields alone, but this relationship is not necessarily a sufficient criterion of the value of a medium. In addition it is desirable to know how media compare with respect to their ability to permit the initiation of growth, and the variability in the yields obtained under controlled conditions. The medium supporting the most abundant growth will not always be found to be best based on these latter criteria.

The presence of toxic factors, unfortunately a frequent occurrence in many of the non-synthetic media in common use, may often be revealed by a decrease in the number of organisms developing from a fixed inoculum as compared to a control medium. In addition the variation in replicate counts will most likely not fit an ideal Poisson distribution when harmful influences are at work. For study of these criteria it may be most convenient to plate out the test organism on the surface of solid media rather than to study growth in liquid media. The number of colonies developing from a fixed quantity of inoculum spread homogeneously over the surface of the plated media and the variation in size of the individual colonies provide



the data for judgment, as well as do the mean sizes of the colonies developed. In comparisons of various media, that medium would be judged the best which permitted the maximum number of inoculum organisms to develop into colonies and showed the least variation in colony size and the largest mean size of colony.

#### MICROBIOLOGICAL ASSAY FOR NUTRIENT FACTORS

Since the growth of a bacterial culture within the range of limiting concentrations of a growth factor is some quantitative function of the concentration of that factor, it is possible to estimate the amount of the factor from the growth response. A large number of microbiological assay procedures based on this principle have been reported with the result that reliable methods for the estimation of amino acids and vitamins are available for research and practical purposes. More recently the principles of microbiological assay have been successfully applied to the development of reliable methods of determining mineral nutrients.

Many growth factors do not have distinctive chemical or physical properties, hence microbiological assays often possess the advantage of being specific when non-biological methods are not. The specificity of the biological method also permits analyses of highly impure preparations. Furthermore, the sensitivity of the microbiological assay may be greater than that of other possible methods. This high sensitivity is especially evident in the case of vitamins whereof the order of one thousandth, in some cases (biotin, B<sub>12</sub>) considerably less, of a microgram per milliliter of sample may be determined with satisfactory precision. The small amounts of sample required are also advantageous since little expensive and scarce material need be consumed in the determination.

When comparisons of the relative concentrations of a growth factor in different samples will provide serviceable information, microbiological assay can be employed for substances of unknown nature. Obviously for such a substance no non-biological method of assay is possible. Thus a microbiological assay procedure is ideally suited for use in studying purification and isolation procedures for new growth factors when strains of bacteria requiring the factors are available.

The technique for all microbiological assays is similar. An organism is put into a medium which is deficient in the essential or stimulatory substance. When increasing amounts of the absent factor are added to the deficient medium inoculated with washed bacilli, the growth response is a direct function of the concentration of the factor added. The growth, or any appropriate manifestation of growth, is measured quantitatively by some convenient means. Increase in cell population, optical density of broth cultures, or metabolite accumulation are most often used. Providing it can

be demonstrated that only one necessary constituent is absent from the basal medium, a substance of unknown chemical structure may be assayed for as successfully as a known substance. The growth response is recorded on a graph, and the resultant curve represents a standard or reference dose-response curve from which the quantity of the factor present in another or unknown sample may be obtained by interpolation. The standard curve should show proportionality between concentration of assay material and the growth response over a practical range of concentrations, and it should be reproducible. However, since some variation is always expected in a biological assay because of the impossibility of controlling all the variables, a standard curve must be obtained for each series of tests or each day's work.

Criteria for the reliability of data from microbiological assays include the following:

1. Values obtained at various assay levels should agree over not less than a four-fold concentration of growth factor.
2. Consistent values should be obtained upon replication at different times.
3. The assay must agree with other possible physical or chemical methods of analysis, and the use of different strains or species of bacteria should yield the same results for the microbiological assay.
4. The method should be specific for the one growth factor under test.
5. Recovery values at various levels of concentration should be within ten per cent of theoretical values. Recovery values are obtained on mixing two sources of growth factor, one known and the other unknown, and should be additive.
6. *Drift* should be absent. Drift is a consistently high or low assay value and is indicative of the presence of interfering substances. Investigations have revealed at least three causes of drift: stimulation by chemically related substances, by chemically unrelated substances, and interference by physiologically related substances.

In the development of a microbiological assay procedure the first problem to be solved is the choice of a suitable basal medium to which the assay material is to be added. The basal medium should be complete except for the assay material. The only limiting variable in the assay should be the growth factor for which the test is conducted. An optimum concentration of basal medium should be employed, this concentration being the one which alone supports no growth or little growth while permitting a maximum response to the presence of the added assay material.

In Figure 67 an example of an actual microbiological assay is given, and Table 36 lists assays which have proven of practical value. Actually for most growth factors it should be possible to devise assay procedures with

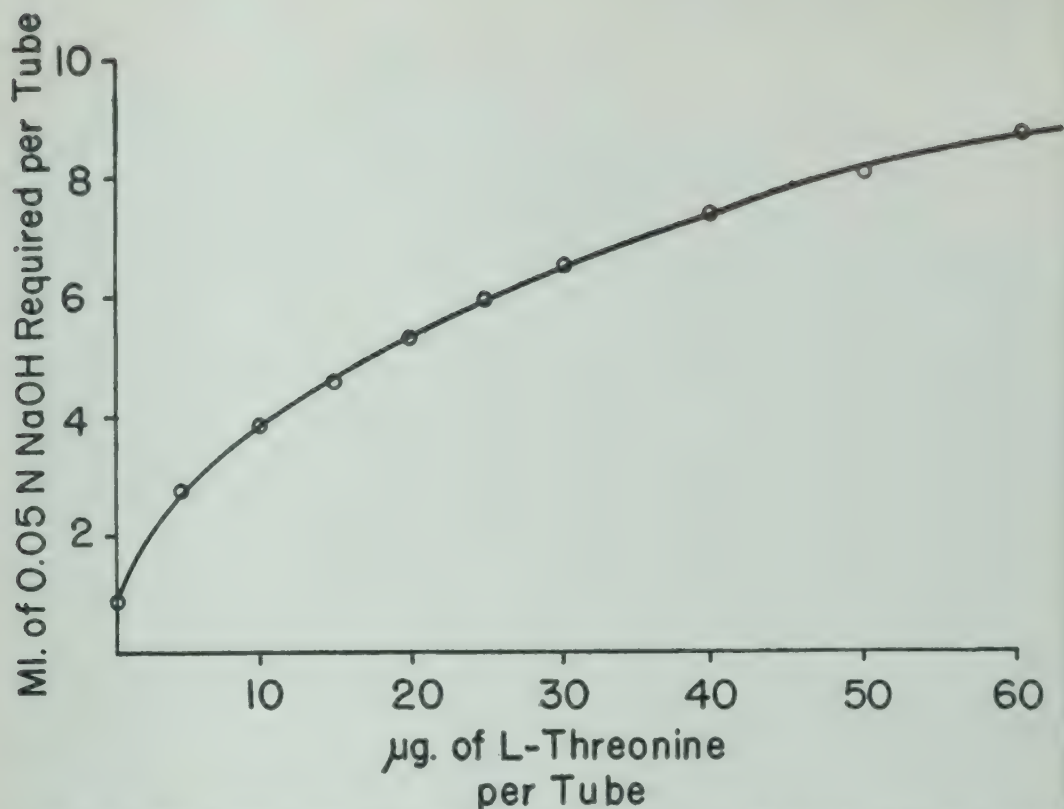


FIG. 67. Microbiological assay for L-threonine in an acid hydrolysate of purified botulinal type B neurotoxin employing *Streptococcus faecalis* as the assay organism. The titratable acidity was determined after incubating 48 hours with the indicated results. The entire contents of each tube were titrated, and the values recorded are averages of duplicate determinations.

Data for standard curve:

L-THREONINE γ PER TUBE	ml 0.05N NaOH REQUIRED FOR TITRATION
0	0.80
5	2.75
10	3.80
15	4.60
20	5.30
25	5.90
30	6.45
40	7.35
50	8.05
60	8.65

γ PROTEIN SAMPLE/TUBE	ml 0.05N NaOH REQUIRED FOR TITRATION	L-THREONINE γ PER TUBE
162	3.25	14.0
324	4.35	13.5
486	5.20	13.0
648	5.95	12.8

13.3

Purified neurotoxin contains  $\frac{13.3}{324} \times 100$  or 4.12% L-threonine.

In the recovery experiment the following mixtures were studied:

MIXTURE	1	2	3
γ L-threonine	5	10	15
γ sample	243	162	81
titrable acidity	5.0	4.85	4.65
γ threonine present			
actual	18.0	16.5	16.0
theoretical	18.3	16.6	15.0
% recovery	98.4	99.4	106.7



TABLE 36

*Bacteriological assay methods for growth factors which have proven of practical value*

FACTOR	ASSAY ORGANISM	PRINCIPLE OF MEASUREMENT	RANGE OF STANDARD
			ml
Vitamins			Zero to
β-Alanine	<i>Corynebacterium diphtheriae</i>	Bacterial nitrogen	1.5 γ
p-Aminobenzoic acid*	<i>Acetobacter suboxydans</i>	Turbidity	10 mγ
	<i>Clostridium acetobutylicum</i>	Turbidity	0.15 mγ
	<i>Lactobacillus arabinosus</i>	Acidity	0.05 mγ
Biotin	<i>Clostridium butylicum</i>	Turbidity	0.1 mγ
	<i>Lactobacillus casei</i>	Acidity	1 mγ
	<i>Leuconostoc mesenteroides</i>	Acidity	1 mγ
Choline†	<i>Pneumococcus Type III</i>	Turbidity	6 γ
Coenzymes I and II	<i>Hemophilus influenzae</i>	Nitrate production	0.037 γ
	<i>Hemophilus parainfluenzae</i>	Turbidity	20 mγ
Nicotinamide	<i>Shigella dysenteriae</i>	Turbidity	0.025 γ
Nicotinic acid	<i>Acetobacter suboxydans</i>	Turbidity	3 γ
	<i>Proteus vulgaris</i>	Turbidity	0.1 γ
	<i>Lactobacillus arabinosus</i> †	Acidity at 72 hrs.	0.1 γ
	<i>Lactobacillus arabinosus</i> †	CO <sub>2</sub> liberated by acid formed at 3 hrs.	13.3 mγ
Pantothenic acid	<i>Lactobacillus arabinosus</i>	Acidity	0.02 γ
	<i>Proteus inorganii</i>	Bacterial nitrogen	1 mγ
Pimelic acid‡	<i>Corynebacterium diphtheriae</i>	Bacterial nitrogen	0.025 γ
Pyridoxine	<i>Leuconostoc mesenteroides</i>	Acidity	0.25 γ
Riboflavin	<i>Lactobacillus casei</i>	Acidity	0.05 γ
Thiamine	<i>Lactobacillus fermentum</i>	Turbidity	5 mγ
	<i>Propionibacterium pentosaceum</i>	CO <sub>2</sub> evolution	0.25 γ
	<i>Staphylococcus aureus</i>	Turbidity	0.5 mγ
	<i>Streptococcus salivarius</i>	Turbidity	0.2 mγ
	<i>Streptococcus faecalis</i>	Turbidity	200 γ
Folic acid			
Amino acids§			
Alanine	<i>Streptococcus faecalis</i>		50 γ
Arginine	<i>Streptococcus faecalis</i>		8 γ
Aspartic acid	<i>Lactobacillus delbrueckii</i>		50 γ
Cystine	<i>Lactobacillus mesenteroides</i>		5 γ
Glutamic acid	<i>Lactobacillus arabinosus</i>		50 γ
Glycine	<i>Leuconostoc mesenteroides</i>		10 γ
Histidine	<i>Leuconostoc mesenteroides</i>		5 γ
Isoleucine	<i>Streptococcus faecalis</i>		10 γ
Leucine	<i>Streptococcus faecalis</i>		10 γ
Lysine	<i>Leuconostoc mesenteroides</i>		20 γ
Methionine	<i>Lactobacillus arabinosus</i>		10 γ
Phenylalanine	<i>Lactobacillus casei</i>		15 γ
Proline	<i>Leuconostoc mesenteroides</i>		8 γ
Serine	<i>Lactobacillus delbrueckii</i>		25 γ
Threonine	<i>Streptococcus faecalis</i>		50 γ
Tryptophane	<i>Lactobacillus casei</i>		2.5 γ
Tyrosine	<i>Lactobacillus delbrueckii</i>		8 γ
Valine	<i>Streptococcus faecalis</i>		10 γ

\* Note that the range of assay of concentrations of a particular factor may vary with the nature of the assay organism.

† For a given organism the useful range of assay may vary with the time of growth at which measurements are made.

‡ Choline and pimelic acid have restricted roles in bacterial nutrition since they are known to be required only by a single bacterial species, the pneumococcus in the case of choline, and the diphtheria bacillus in the case of pimelic acid.

§ Note that in general the range of concentrations for the assay of amino acids is considerably higher than for the vitamins. This is probably a reflection of the fact that the amino acids are converted into a major quantitative constituent of the organic matter of bacteria. Vitamins are coenzymes or precursor materials for the synthesis of coenzymes and as such are required in very small amounts.

|| With glutamic acid the assay curve obtained is sigmoid in shape and unlike that of Figure 67. On the other hand a normal non-sigmoidal assay curve is obtained for glutamine. If ammonium salts are omitted from the basal medium for the assay of glutamic acid, the curve of growth response shows a still greater threshold dose. These results have been interpreted as meaning that glutamic acid as such is not utilized by the assay organism but must first be converted to glutamine.

The data in this table are based upon information in Peterson and Peterson (1945), and Snell (1948), and on personal experience.

other organisms than those listed. In general where a choice has been possible the lactic acid bacteria have been favored by students of microbiological assay. There are a number of reasons for this preference. Growth response may be measured either by turbidity or the titratable acidity which develops in the medium containing glucose. These organisms do not tend to autolyze as readily as some other bacteria so that if growth response is measured beyond the logarithmic period of growth the complicating effects of autolysis are minimized. Furthermore, these organisms need numerous growth factors which makes it necessary to carry only a few different strains in a stock culture collection for use in assaying a wide variety of substances.

It will be instructive to consider briefly the data of the microbiological assay recorded in Figure 67. Replication in the assay was excellent since titration values of duplicate determinations were within 0.1 ml. of each other. The recovery values appear to be within ten per cent of theoretical values. Yet in spite of these apparently good results the assay cannot be considered to be reliable. Note that in the standard curve there is no linear relationship between the amount of threonine added and the amount of acid produced in the culture. As the amount of threonine is increased there is relatively less increase in acid production. From this one suspects that threonine is not acting as the only limiting factor for growth and acid production. As a result the assay values of the unknown sample show a consistent drop as the amount of sample per tube is increased. The recovery values show the same tendency.

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## The Variation and Genetics of Bacteria

Of all organisms bacteria, minute in size and lacking the complex processes of differentiation associated with the embryologic development of multicellular organisms and their specialized tissues, might be expected to exhibit the least variability. This hypothesis is justified if for no other reason than the total number of apparent properties that could possibly vary are fewest in these smallest of organisms. Nevertheless an intimate acquaintance with bacteria reveals a full capacity for variation by the characters they do possess.

This capacity for change has been so impressive as to have fostered at one time in the early history of bacteriology a school of thought, *pleomorphism*, that was skeptical of the existence of any but a single or perhaps a few species of bacteria having a protean capacity for variation. Yet with the development and the application of strictly controlled methods for obtaining pure cultures and with the realization of the constancy with which certain types of bacteria could be isolated from natural environments, a totally contrary view arose, *monomorphism*. This view admitted the existence of only the most meager potential for variation by individual bacterial species and in its extreme form would base the taxonomic separation of species on single character differences.

Depending on which observations have particularly impressed each generation of bacteriologists, monomorphism and pleomorphism have had fluctuating fortunes of acceptance. In this case as has happened so often in the history of ideas, the human tendency to set up opposing dichotomous propositions has proven of false value in offering true explanations of complex and poorly understood phenomena. The truth is generally neither all black nor all white and mayhap lies in ignored directions.

Our understanding of bacterial variation, though having its roots in the distant past, has made its greatest progress in the years since 1940 and has come primarily from the application of the general ideas of genetics. The concepts of pleomorphism and monomorphism and the specialized vocabularies to which they gave rise are being gradually replaced by those of modern genetics.

Historically it would seem that a true understanding of bacterial variation has awaited and been dependent upon the prior evolution of genetics to an applicable stage of development. If the applications of the ideas and

techniques of genetics to the problems of variation in bacteriology continue to be as productive as they have been, and they show every prospect of so being, we shall finally witness a complete synthesis of the data of bacterial variation into the general framework of genetics. A concomitant increase will occur in the investigations employing bacteria as the means for the study of certain fundamental problems of genetics.

### THE OPPORTUNITY FOR BACTERIAL VARIATION

The significant time scale for genetic experience is the generation time or the rate of multiplication. Relative to the life span of the human observer, opportunity is afforded for the observation of events covering an enormous number of generations of bacteria. In addition, at any one time there exists an uncountable number of individual bacteria, and the observer may readily study untold billions of these organisms within the narrow confines of his equipment and laboratory. These facts provide an opportunity for noting variations among bacteria unparalleled in the experience with larger organisms. Certainly the probability both for the occurrence of individuals differing in the extreme from the average and the opportunity for detecting them is a function of the number of generations and total number of individuals scanned. Therefore, it is not surprising that one may come away from a study of bacteria marveling at the extraordinary variability they seemingly possess. Yet when the proper comparison is made with other living species and account is taken of the differences in generation times, the sizes of natural populations, and the absolute numbers observed, it is doubtful that bacteria are any more inherently capable of showing variation than other organisms. This conclusion is fortified by the knowledge that the measurable mutation rates of bacteria are of the same order of magnitude as for other organisms.

The characteristics of an organism are inherent in its genotype, but the phenotypic expression of a genotype is not an absolutely fixed event. The genotype is the hereditary constitution of an organism. The phenotype is the actual appearance or the physical expression of the genotype of the organism. The genotypic substratum is sufficiently plastic to permit a range of variation in the phenotype depending on the nature of the environment and the interaction of the organism with its environment. It has often been said that the organism is free to develop or adapt in response to its environment within limits set by the genotype.

In this regard, bacteria are probably no different than other organisms except for their unusual opportunities of meeting with environments of diversity within which their development must take place. Consider for a moment the remarkable variety of experiences an individual bacterium may undergo within a short time in its natural environment. The organism in the



sap exuding through a cut in the bark of a maple tree has been exposed in a single hour to a great variation in temperature, humidity, osmotic concentration, and radiant energy even if it has escaped the intestinal tract of some passing sweets-loving animal.

While the macrocosm of man's existence may appear relatively stabile, it is made up of innumerable microcosms of bacterial existence having great differences. The bacterium in a particle of soil directly exposed to the winds and light of the atmosphere is living in a radically different environment than some relation deposited by circumstance in the dark and possibly anaerobic soil below the surface. The bacterium buried in a mass of colonial growth, immediately bathed by the excretory products of its pressing neighbors, and feeding on the remnants of nutrients which have somehow successfully diffused through a mass of avariciously metabolizing sister organisms is living in an entirely different world than the fortunate bacterium a short distance away directly exposed to the nutrient agar at the edge of the colony.

This diversity of environment in which phenotypic expression of the genotype must occur is also emphasized for bacteria by their small size and acellular nature. A great part of their body mass is in direct contact with the external world. In contrast, and in conformity with the laws of geometry, larger organisms and multicellular forms have relatively less of their mass exposed to direct contact with the outside world. Most of the cells of multicellular organisms are not exposed at all and live continuously in a self-contained and regulated internal environment.

The multiplicity of environments to which bacteria are exposed increases the opportunity for the development of the variety of phenotypes permissible within a genotype. It also places a positive survival value on changes in genotype which otherwise might be lethal in more monotonous and changeless environments. Thus the lactose fermenting bacterium growing in cow's milk which mutates to a non-lactose fermenting sport does not lose its opportunity to perpetuate its protoplasm since in the natural course of events its progeny may end in the intestinal canal of a suckling calf. The calf will "thoughtfully" hydrolyze the lactose to constituent monosaccharides which could be utilized by the bacterial sport. How different the fate of the ill-favored cow who must witness the demise of any calf unfortunately born without the capacity to utilize lactose.

The perpetuation of a change in the genotype of bacteria is also favored by their predominantly asexual mode of reproduction. The bacterium which undergoes a change in genotype transmits this change to its immediate progeny. Thus unless the change is lethal, it is preserved. However, the situation with sexual reproduction is not quite so favorable for perpetuation of genotype changes. Changes in somatic cells cannot be passed on by

sexual reproduction no matter what their survival value and are lost upon the death of the individual organism in which they occur when the individual is not capable of an asexual as well as a sexual method of reproduction. Only changes occurring in sexual cells can be transmitted, and these may be lost unless the gene or chromosome with which the variation is associated is transmitted during meiosis to a viable gamete. Even passing this hurdle does not guarantee the continued existence of the changed genotype since fertilization may not occur, a highly likely event in most species particularly when the change is carried by the male gamete or sperm.

### THE KINDS OF BACTERIAL VARIATION

Variations have been described as *continuous* or *discontinuous*. A character which varies solely in a quantitative way is *continuous* if no sharp breaks may be discerned among individuals showing such variation, the extremes of difference being connected by a series or even a continuous spectrum of the possible intermediate properties. A typical example of this type of variation is the difference in size exhibited by bacteria within a clone.

A *discontinuous* variation may be one of a qualitative nature and consists of relatively easily catalogued differences or is a quantitative change which may be discerned to fall into clearly separable groups. The gain or loss of the ability of bacteria to ferment a particular sugar or to produce hydrogen sulfide would be examples of discontinuous variations.

It is recognized that a character subject to discontinuous variation can also vary in a continuous manner but that the alternative is not always possible. Thus bacteria may differ in their absolute ability to produce hydrogen sulfide, and those which are able to produce the substance will also vary in the rates at which they do so. All the possible rates of production by single bacteria will probably be found in the range between the most and least actively acting organisms. On the other hand, any organism must have size, so size as a characteristic can only change quantitatively.

The designation of variations as continuous or discontinuous is useful since it suggests the means which may be applied to discover and study variation. Unfortunately, however, variations which have different causes are lumped together, and it cannot be stressed too strongly that the chief interest of bacteriologists in the problem is to understand the basis for the variation observed. A mere recording of facts cannot be scientifically satisfying. The accumulation of descriptive data is necessary, but it is only the first effort in formulating a classification based on the identification and separation of variations according to their causes. The following is one such possible list of variations.

*Genotypic Changes.* These have also been called permanent and transmis-



sible variations. Until definitely proven cases of cytoplasmic inheritance are found in bacteria, all genotypic changes are referred to nuclear changes.

- A. Chromosome mutations or breaks, inversions, translocations, losses, polyploidy. In the present state of cytological knowledge of the nuclear apparatus of bacteria it is not possible to know with certainty if these events occur and how important a contribution they may make to observed cases of transmissible variations. In this connection it is of interest that colchicine, a substance known to interfere with the normal picture of mitosis, has no detectable effect on bacterial variability.
- B. Gene mutation. Most genotypic changes observed with bacteria seem to be analogous to the phenomenon of gene mutation as observed in other organisms.
  - a. Dissociation. Characteristic changes in cultural characteristics of bacteria associated with genotypic changes which are studied chiefly as changes in the morphology of bacterial colonies on solid media.
  - b. Secondary colonies. The growth of discrete colonies within the confines of another colony after the major growth of this original or primary colony has been completed. The secondary growth differs in one or more properties from the parent strain.
  - c. Sectors. Discrete portions of colonies differing in some discernible character from the parent strain, growing simultaneously and competing successfully with the parent type. The formation of a sector may be favored by a gene mutation early in the development of the parent colony. They may be due to gene recombination in a sexually reproducing heterozygous population, to segregation from heterozygous diploids, or to segregation of haploid uninuclear bacilli from multinucleate organisms.
- C. Segregation. In sexual reproduction the reduction division of meiosis followed by the independent assortment of chromosomes and recombination during fertilization provide opportunities for the existence of hybrid genotypes impossible of origin by asexual mechanisms of reproduction.
- D. Transformation or infective transmission. Under appropriate conditions the addition of desoxyribonucleic acid from a given strain of a bacterial species to a growing culture of a different strain of the same species may induce a change in the exposed strain to the type from which the transforming desoxyribonucleic acid was derived. This phenomenon was first noted and has been most thoroughly studied in the case of transformations of the pneumococcus capsule type.

*Developmental or Temporary Changes.* These variations occur in response to the nature of the external environment, the physiological state of the internal environment, and their interactions. It must not be forgotten that



these variations are phenotypic responses to environment somehow conditioned by the genotype. Whether or not these variations are orderly in sequence, and repeated in different generations (*cyclic*) depends on the recurrence of the factors inducing the response.

- A. Cytomorphosis. The changes in bacterial morphology and associated physiological variations due to the stage of the growth cycle of the culture.
- B. Fortuitous variation. Normal distribution of variation of a character within a population. When any particular character of a biological population is measured, even of individuals within a clone, a continuous variation described by some normal distribution curve is found. Individuals do not appear exactly alike in spite of similar genetic backgrounds since the seemingly uniform environment from which a biological population is obtained for study is actually variable in its parts. The small differences between the individuals arise from a great many largely indeterminate causes, no one cause predominating.
- C. Fluctuation or physiological variations. These are the effects of the physical and chemical environment on the metabolism, growth, and multiplication rates and the ratios existing between these activities. Included would be variations due to changes in the nature and quantities of utilizable foods, pH, oxidation-reduction potential, interfacial tension, osmotic concentrations of the culture system, and responses to toxic agents.
  - a. Adaptive enzyme formation. This would be a special case of physiological variation.

*Involution.* Dead or dying bacteria in a culture may present different appearances than normally metabolizing and multiplying organisms. The action of autolytic enzymes restricted in their activity in living organisms contributes to the production of unusual forms. Such abnormal looking bacteria are labelled involution forms. Since direct observation of bacteria does not permit a reliable estimate or separation of the dead or dying from the living, the characterization of a particular case as an involution is not always an easy task. Yet, since they have little biological significance, it is important not to confuse involution forms with other types of variants.

Difficulties in methodology are involved in arriving at a decision regarding the origin of a variation as a genotypic change. The problem is not simple since we must depend upon a phenotypic expression to indicate the change in genotype. The solution rests on being able to determine the quantitative relationship of the number of organisms exhibiting the variation to the numbers exposed to the environment in which the phenotypic character is observed. The logic of this approach rests on the obvious principle that if a variation is merely a developmental response to a particular

environment then the response should be dependent only upon the opportunity for exposure of bacteria to the same environmental influence and should be completely independent of the number of individuals so exposed. Contrarily, within a bacterial population in which genotypic changes are taking place only those individuals arising from a parent of changed genotype will respond to a test environment by a change in phenotype. The proportion of such individuals in a population will be a function of the rate at which the change in genotype occurs and the relative survival values of the new and parent genotypes. A test environment is chosen in which a uniform phenotypic response by the differing genotypes would not be expected.

To decide whether transformation is involved in cases of variations is not a difficult task. The necessary transforming role of desoxypentosenucleic acid suggests a specific test for inhibition of the induction of variation. Specific enzymes such as the hydrolytic desoxyribonucleases are employed to inactivate the transforming nucleic acid.

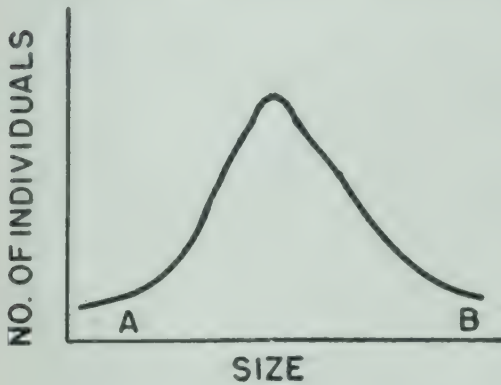
#### GENOTYPIC VARIATION

To understand hereditary changes and their consequences in bacteria the concept of the pure line and that of selection must be kept in mind. While genetic variations provide the opportunity for evolution, the processes of selection determine the changes which can survive. In a developing bacterial culture there is enacted on a small scale all the drama of evolutionary change with its accompanying rise and fall and shifting of populations. The materials of bacteriological studies ordinarily consist of populations, and insight into bacterial processes entails some knowledge of this picture of population dynamics.

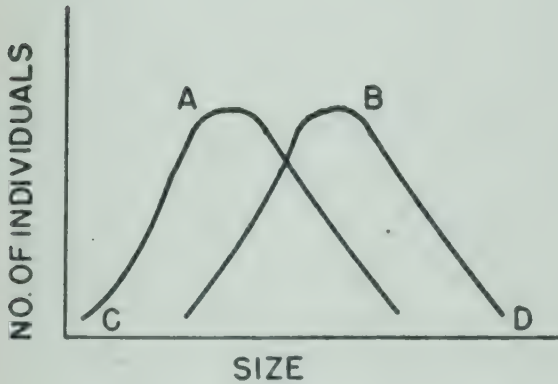
A *pure line* is all of the individuals descended from a common ancestor and possessing an identical, inherited constitution. Thus when no genotypic variation occurs all the individuals in a clone are members of the same pure line. The individuals of different clones originating from a common ancestor also all belong to the same pure line if a genotypic variation has not intervened. When a variation of genotype does occur within a pure line then all the individuals descended from the genotypic variant constitute a separate pure line. Intrinsic in the concept of the pure line is the fact that any variation within a pure line must be developmental and traceable to the complex of environmental factors with which the genotype of the pure line interacts.

*Selection* is the act of choosing and is ineffective as a means of changing the characteristics of a population when applied within a pure line. Selection can affect the inherent biological characteristics of a population only by exercising a choice among individuals of varying genotype (fig. 68).

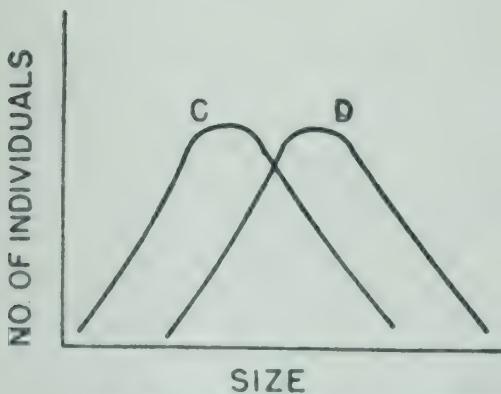
Ultimately hereditary differences between individuals owe their origin to gene mutation. Presumably the genotypic differences characterizing species as well are made possible only because at some time in the past



HETEROGENEOUS POPULATION



POPULATIONS DERIVED FROM  
SELECTED INDIVIDUALS A AND B



POPULATIONS DERIVED FROM  
SELECTED INDIVIDUALS C AND D.  
SINCE PURE LINES HAD ALREADY  
BEEN SELECTED IN THE FIRST  
STEP FURTHER SELECTION IS  
INEFFECTIVE.

FIG. 68. Illustration of ineffectiveness of selection within a pure line

gene mutation or differentiation led to a chromosome structure and finally to chromosomes of differing gene composition.

By present day standards the great bulk of variations of the genotype met in bacterial cultures are due to currently occurring gene mutations



Chromosome changes other than those involving gene mutation are in doubt, and recombination and transformation seem to be quantitatively much less frequent than gene mutations. For bacteria, therefore, selection can be thought of as predominantly a process of choosing among gene mutants.

The growth habits of the bacteria, environmental factors, and the interaction of these, as well as the mutation rates, determine the relative proportions of mutants present in a population at a given moment. The final composition of the bacterial population in a culture in terms of the number and relative proportions of pure lines present is the result of selection. The selection operates through mechanisms involving the differential viability and growth rates of the pure lines.

Any property of an organism has a survival value large or small. The survival value of a property or of a change in genotype, however, is not an absolute quantity; rather it is related to the nature of the environment. To illustrate: mutation of a non-nitrate reducing *Bacillus vulgatus* to a nitrate reducing genotype will have no positive survival value when the organism is growing in the large intestine of man which is practically free of nitrates but may have a distinct value for the organism growing in a nitrate rich soil.

#### MUTATION

Colloquially a mutation is defined both as any sudden change or as the appearance of offspring differing in some well marked way from the parents. In genetics only transmissible variations are spoken of as mutations, and these may be caused by chromosome mutations or gene mutations. Inasmuch as most genotypic changes in bacteria simulate gene mutations, the word mutation is most frequently used in the bacteriological literature to signify a gene mutation and is so employed in this text.

The identifying characteristics of gene mutations are several:

1. Sudden. No forewarning of their appearance is given; their appearance is abrupt.

2. Spontaneous. The natural appearance of mutations is spoken of as spontaneous since their occurrence is unpredictable and completely random. Nothing that is known can be done to favor the occurrence of a particular mutation. Mutagenic chemicals such as the nitrogen mustards and physical agents such as radiant energy affect only mutation rates, for the induced mutations are of the same kinds as spontaneous ones.

3. Relatively independent of environment. In nature temperature is the only factor which can be consistently shown to influence spontaneous mutation rates. With the bacterium *Phytomonas stewartii* temperature coefficients for mutation of 2.5 to 5 have been recorded. Certain characters

become very mutable at the maximum temperature permitting growth. A probable corollary of the influence of temperature is the relatively greater abundance of different species and varieties of plants and animals in the tropical than in the temperate zones of the earth. A contribution to the greater variety of forms in warm climates may also be made by the larger populations and shorter generation times which would provide for a greater absolute number of mutations even when the rates of mutation are fixed.

4. Constant rate of occurrence for a given set of conditions.

5. Permanent change. This criterion should not be interpreted in an absolute sense since *back mutation* (*reversion*) of a gene to its original state may take place, as well as a second mutation of the gene to still another new state (*forward mutation*).

Mutation, like other characteristics of organisms, seems to be itself subject to some sort of genetic control, as observed in *Drosophila* and other sexually reproducing organisms. Thus, hybridization has been found to affect the mutability of certain genes. In *Drosophila* a mutator gene has been detected which by its presence is capable of increasing by ten-fold the mutation rate of other genes. So impressive are the indicated effects that mutator genes have been hypothesized to be the major cause of spontaneous mutation. While such genes have not been described as yet in bacteria it would be surprising if they did not occur.

Mutation affects only a single gene at a time, and the spontaneous mutation frequencies of individual genes in bacteria have been recorded in the range from  $10^{-10}$  to  $10^{-3}$  per bacterium per division with most being at the smaller rates. The differences in the spontaneous mutation frequency between various genes of bacteria are greater than can be accounted for by chance alone as is also true of other organisms. This means that some genes act as though they are inherently more mutable than others, possibly suggesting that mutator genes do not affect the mutation rates of all genes to the same extent.

Apart from the suggested role of mutator genes the cause of spontaneous mutation is unknown. The rates cannot be attributed to incident radiation in the natural environment. The natural radiation could only account for one per cent or less of the observed rates. In addition mutation induced by radiation is independent of temperature while spontaneous mutation is not.

Mutation may be lethal. Wild species display less variability than domesticated species, and fewer mutants appear able to establish themselves in a wild population. Since wild populations have evolved by a process of selection from mutating natural populations it is not surprising that most mutations appear to be harmful or even lethal. An existing genotype of the wild variety tends to be that best suited to the particular environment and remains fairly stable.



A lethal mutation is not necessarily fatal in all environments. Thus, mutation of a strain of *Escherichia coli* to a requirement for tryptophane as a growth factor will be fatal when the organism is growing in a glucose-ammonium salt medium in the laboratory but not lethal when the organism is in the intestinal tract of man where some tryptophane is probably present and can be utilized for growth.

### *Calculation of Mutation Rate*

Spontaneous mutation seems to occur only in bacteria undergoing growth and multiplication. Available evidence, still too scanty to permit generalization, indicates that mutation rates are not a function of the multiplication rate. At a constant temperature if the concentration of a nutrient factor is varied to cause a change in multiplication rate, the mutation rate remains constant per bacterium per unit time. Therefore, an appropriate unit for expressing mutation rates is the number of mutations per bacterium per unit time.

Other units widely used are the number of mutations per bacterium per fission and the number of mutations per bacterium per division cycle. The former unit implies that mutation occurs only at the time of division, while the latter unit assumes that the mutations occur throughout the period of growth from one fission to the next and at rates related to the fission rate. These units should be superseded by the first mentioned unit if extended experience with different bacterial species indicates a general correlation of mutation with time during growth rather than with generation time.

Critical discussions of the details and estimation of mutation rates are provided by Luria and Delbrück (1943), Lea and Coulson (1949), Newcombe (1948), and Lederberg (1951). A method is illustrated in Figure 69.

### *Delayed Expression of Gene Mutation*

Study of the phenotypic expression of the occurrence of both spontaneous and induced gene mutations in bacteria reveals the existence of two kinds of mutations, *zero point* and *delayed* or *end-point*. A zero point mutation is characterized by immediate phenotypic expression of the change of genotype in the bacterium in which it has occurred. The fission of the bacterium after a zero point mutation results in progeny both showing the dependent phenotypic change. Delayed or end-point mutation is one having no immediate phenotypic expression in the mutated bacterium (figure 70). The organism must divide one or more times before the mutation is expressed phenotypically, nor need all the progeny of the mutant show the changed genotype. The phenotypic expression of a spontaneous delayed mutation usually takes place within the first few fissions after the occurrence of the mutation and is complete by the sixth fission. With induced delayed muta-



tion phenotypic expression tends to be more frequent beyond the first few fissions of the mutated organism and may be postponed for as long as thirteen divisions. Whether this indicates a difference in the nature of spontaneous and induced end-point mutations is unknown.

Steps:

### U Culture as a source of inoculum.

1. Small and equal quantities of inocula are transferred to a series of tubes containing only a small volume of nutrient broth.



2. Tubes are incubated until growth is completed whereupon the number of organisms within each culture is determined by some convenient means.
3. Content of each tube is transferred to a selective and, or differential medium on which mutants will be distinguishable from the parent type.



4. Mutation rates are calculated from the percentage of tubes showing the absence of mutants and the average number of bacteria present in the tubes:

Let  $p_0$  represent the fraction of tube cultures in which no mutants appear,  $N$  the average number of bacteria in the cultures at time  $t$  when the contents of the tube were transferred to the selective medium in the petri dishes, and  $\ln$  natural logarithms, then

$$\frac{-\ln p_0/N}{t} \text{ is an estimate of the number of mutations/bacterium/unit time}$$

or

$$\frac{-\ln p_0}{N} \text{ is an estimate of the number of mutations/bacterium/division}$$

or

$$\frac{-(\ln 2)(\ln p_0)}{N} \text{ is an estimate of the number of mutations/bacterium division cycle}$$

FIG. 69. A method for determining mutation rates

In the method illustrated a small inoculum is employed to seed each of the tubes in the series in order to reduce the chance for accidentally including a mutant in the inocula. Since the method provides an estimate of the number of cultures which show mutants, the addition of a mutant to any tube in the series would bias the estimate of the mutation rate and yield a value higher than the true one.

The purpose in using only a small amount of medium (0.1-1ml.) in each culture tube is to avoid the possibility of mutation occurring in all or most of the tubes, a likelihood which is increased with an increase in total population (absolute number of organisms in a tube).

From the nature of the method it is obvious that the reliability of the estimate of mutations is increased by employing larger numbers of tubes. In this regard the statistical problems of the method are similar to any method of estimate based on a *most probable number determination*. A further limitation is the ability to observe experimentally all of the mutations which do occur.

The technic for detecting delayed mutation is not difficult if a selective medium or other means for selection are available for separating a mutant from the original type. For example, bacteriophage has been employed to detect a mutation from sensitivity to resistance to lysis by the bacteriophage. The surfaces of a series of plates are seeded with equal quantities of a test population of bacteria susceptible to phage and immediately afterwards the organisms are exposed to say, x-rays which is a mutation inducing agent. An irradiated plate is immediately sprayed with bacteriophage and

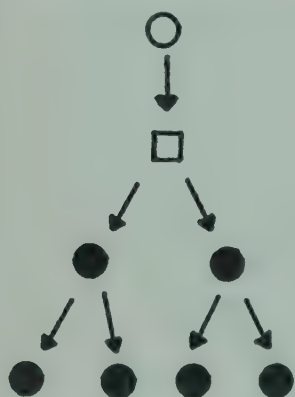
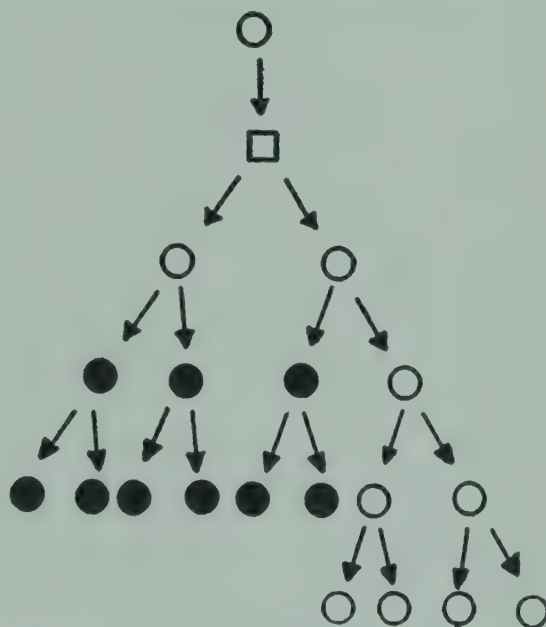
**ZERO POINT MUTATION****END POINT MUTATION**

FIG. 70. Illustration of zero point and end point mutation. The example for the delayed type is only one among the many possible cases. ○ = Normal type phenotype. ● = Mutant phenotype. □ = Mutated bacterium.

incubated. The other plates are incubated for varying lengths of time to permit the development of microcolonies before being in their turn sprayed with phage and reincubated.

Visible colonies developing on any of the plates originate from mutant organisms resistant to lysis by the phage. Colonies appearing on the plate that was sprayed with phage without prior incubation represent growths from zero point mutations since the organisms were exposed to phage without any opportunity for their multiplication before the phage was added. The colonies on the other plates represent growth both from delayed and zero point mutations. Hence the difference in the number of colonies on these plates relative to the plate with the zero point mutants alone

represents colonies originating from organisms showing phenotypic lag. The differences among the plates preincubated before exposure to the phage can be used to estimate the number of fissions required for the delayed phenotypic expression of mutation. Taking into account the generation time of the organism this can be done by relating the differences in number of colonies to the differences in the lengths of the incubation times for micro-colony formation before spraying on the bacteriophage.

A full explanation of the zero point and delayed expression of mutation is not yet available although numerous possibilities present themselves of which a few may be mentioned. If bacteria are diploid, zero point mutation could represent simultaneous mutation of an allelic gene pair, an unlikely explanation since it is not in accord with the high frequencies at which zero point mutations occur. In fact the order of magnitude of observed mutation rates and the frequency of zero point mutation favor a hypothesis postulating the general existence of the haploid state among bacteria and haploid nuclei both in uninucleate and multinucleate bacteria. The existence of multinucleate organisms from which mutated nuclei segregate would explain the phenotypic lag if phenotypic expression occurred in the first few divisions beyond the occurrence of the mutation. But this seems not to be true for induced delayed mutation in which case most of the delayed phenotypic expression generally occurs after the first few fissions and as late as the thirteenth.

The phenotypic expression of the mutation of a gene might wait upon the exhaustion of the normal somatic or cytoplasmic substance already present in the mutating organism. It would take time representing the growth and multiplication of the organism before post-mutational somatic material resulting from the changed synthetic capacity of the mutated organism could replace the original cellular substance. This hypothesis has been labelled that of *phenomic delay*. A difficulty with this view is that within a few generations removed from the mutated parent all the progeny would be expected to show the changed phenotype, but this has not been shown definitively. From the available data it would appear that some progeny of the organisms showing end-point mutations retain the original genotype. Then again while phenomic delay may explain delay, it does not explain the zero point mutation of apparently the same gene in other individuals of the same pure line in which the end-point mutation occurs. Can this be explained by merely assuming the zero point mutation to occur in a bacterium with practically no pre-mutation products to be exhausted while sister cells have variable quantities of these same materials which require, two, six, or even thirteen fissions before they are replaced by post-mutational products of synthesis? This does not seem to be probable.



While the gene at a given locus on a chromosome is spoken of in the singular, it may well be that gene reduplication in bacteria is of such a nature that a gene is actually represented several times at a given locus. Depending on whether these mutate simultaneously or independently a zero point or end-point mutation might be expected.

This brief consideration of a complex problem merely points up the work to be done and the exciting possibilities for expanding fundamental knowledge of cytogenetics in arriving at a solution for the problems raised.

### *Nutritional Mutations*

So large a volume of the work in bacterial genetics has involved the study of nutritional mutations that these will be discussed separately. A part of the interest in studies of these mutants is due to their value as means for attacking problems of intermediary metabolism. Nutritional mutations may be of a kind resulting either in the need for a specific nutrient because of a loss in synthetic capacity, or contrarily the acquisition of independence of the need for a particular nutrient due to a gain in synthetic capacity. The latter type of mutant is easy to isolate since it alone will grow in a medium deficient in a nutrient factor that is required for the growth of the parent strain.

One ingenious method for isolating mutants with a decreased synthetic capacity is to seed populations of the parent strain into a medium deficient in the nutrient factor required by the desired mutant but containing penicillin at a bactericidal level. Since penicillin acts only on growing bacteria the parent type which can grow on the nutrients provided will grow and be killed. Survivors will be the biochemically deficient mutants which can be isolated by plating out the penicillin "sterilized" culture on a penicillin-free complete medium containing the needed growth factor. Another procedure involves spreading a population on a deficient agar medium and incubating. The parent type will grow out to form visible colonies. The missing growth factor is then added to permit the growth of the deficient mutants. Colonies of these mutants will grow out in the now complete medium but will be distinguishable from the parent type by their smaller size.

Subsequent to a mutation to a state of deficiency in synthetic capacity a reacquisition of the lost synthesis may be observed. This may be either a true reversion or a forward mutation resulting in the synthesis of a product by an alternate metabolic path. It is often desirable to decide between these possibilities. The mutability of a true back mutant should be the same as that of the original parent since the two should be identical in all respects. If more than one phenotypic change characterized the muta-

tion, a reversion should restore in a single step all of the phenotypic characters of the original parent.

Any mutation suppressing the synthetic ability of bacteria is a potentially lethal mutation, for it will be lethal whenever the essential products of the lost synthesis are absent from the environment. The presence of a limiting product in the environment may depend on the existence of a complex flora. Hence in mixed cultures the excretion of the required material by some organisms could mask the lethal nature of the mutation. The more varied the flora present the greater is the chance for this to happen.

*Satellite colonies* are those of biochemically deficient organisms growing in the immediate presence of other colonies from which a synthesized product diffuses and which is the required growth factor that the satellite organism is unable to manufacture for itself. A related phenomenon is *syntrophism* which is the development of clones of distinct mutants in a single culture as a result of the ability of each clone to synthesize and release the growth factor required by the other. If mutations represent blocks at different steps in a catenary series, syntrophism is presumably accounted for by the interchange of precursors (fig. 71).

The term *prototroph* is applied to a strain of a species originally unlike the wild type in nutritional requirements and which has reacquired the characteristics of the wild type by mutation. The strict and older meaning of the term is any primordial organism with respect to nutrition. In the *Enterobacteriaceae* the strains capable of synthesizing necessary growth factors are generally considered to be the primitive type, hence the logic in calling prototrophic the mutation of any strains requiring growth factors to strains independent of the need for an external supply of those growth factors. Though not abundant in number, strains of *Enterobacteriaceae* requiring growth factors are found in wild populations. Apparently a reduced capacity for anabolic synthesis does not always place these organisms at a disadvantage in nature.

In addition to absolute gains or losses in the ability to synthesize substances, mutations may result in a quantitative change in enzyme activity and in the quantity of a product accumulated. This increase may be due to a real increase in the rate of production of the product or to an accumulation resulting from blockage of the metabolic utilization of that substance. Furthermore, increased accumulation of a substance may be due to an *anapragmic* mutation, one resulting in increased enzymatic activity by means of the removal of an inhibitory influence.

### *Dissociation*

The term dissociation has been applied to transmissible variations in the appearance of cultures of bacteria and particularly to changes in colonial

morphology. Conversely colonial morphology has been employed in devising nomenclatural schemes for use in the identification of dissociants. Smooth or S type denotes a raised colony with regular edges and a surface that

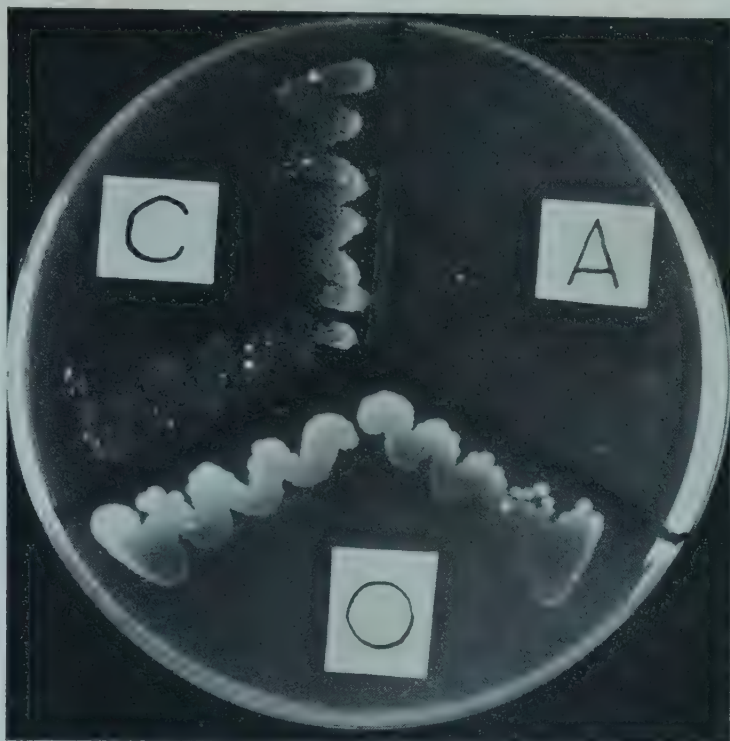


FIG. 71. Arginine syntrophism (on 0.01% NZ medium), an example of syntrophism in *Escherichia coli*. The wild type strains with an unimpaired synthetic capacity are able to grow in media deficient in arginine and its precursors. However the mutant strains with which the illustrated petri dish is seeded are unable to synthesize arginine since the precursor reactions involved in arginine synthesis are genetically blocked at different stages. Mutant O grows in response to ornithine, citrulline, or arginine; C responds to citrulline or arginine; and A responds to arginine only.

In the N-Z medium on which the mutants are seeded, a limited quantity of arginine is present. Thus a limited amount of growth of the three mutants is possible. However during growth A excretes ornithine since these precursor materials cannot be synthesized into arginine by the mutants. As a result these substances become available as growth factors for the other mutants utilizing them. The relative abundance of growth is a measure of the difference in the total quantity of growth factors available to the mutants, and the gradients of growth reflect the diffusion gradients of utilizable growth factor arising from the genetically blocked mutants.

(Reproduced by Courtesy of B. D. Davis)

appears homogeneous. Rough or R type identifies flat colonies with irregular surfaces and edges. Mucoid or M colonies are glistening smooth colonies exhibiting a gummy or viscid consistency when manipulated with a platinum loop. The M colony is associated with the production of definite



capsules or large quantities of viscid slime layer material. All sorts of colonial types intermediate in appearance have been described as well as more infrequent types such as phantom (P), gonidial (G), and diphtheroid (D). Various common appearances of colonies and a suggested nomenclature for these appearances are illustrated in Figure 72.

The appearances of bacterial cultures are a reflection of their morphology. The tendency of bacteria to grow in chains or to separate after fission and their surface characteristics in general are the basic properties which determine the picture of their massed growth. The nature of the medium can also affect the appearance. A broth medium of low surface tension might result in diffuse growth of a particular organism, while one of high surface tension would permit pellicle formation by the same organism. An agar medium with five per cent agar and little free water at the surface would tend to give compact colonies smooth in appearance whereas a one per cent agar might yield a spreading colony with a granular surface. The term dissociation is not meant to apply to these developmental variations that result from a response to environment but is limited to appearances which persist on subculture and which are not merely characteristic for a specific environment.

Dissociation usually involves a number of changes in the properties of an organism (Table 37). While the cultural picture has provided the foundation for a nomenclatural scheme for purposes of identification, there are often simultaneous changes in antigenicity and in the biochemical activity of the organism. With pathogenic and toxin producing bacteria the smooth types are generally the more virulent and the better producers of toxin.

There is good reason to believe the origin of dissociants to be by means of gene mutation. Mutation of a pleiotropic gene would result in changes in the number of characters directly affected by the gene. Apart from this possibility the complex of changes generally observed in dissociation could in part, if not *in toto*, be accounted for by numerous indirect effects following a change in a major property of the organism. A proven instance of this situation is the loss of virulence exhibited by those mutants unable to synthesize purines and *p*-aminobenzoic acid and which are derived from wild strains of *Salmonella typhosum* virulent for mice. The body fluids of the mouse contain only low levels of free purines and *p*-aminobenzoic acid so that the *in vivo* growth of the mutants deficient in purine and *p*-aminobenzoic acid is limited. The injection of quantities of the limiting nutrients into mice results in the *in vivo* growth of the bacilli to toxic levels. In this instance it is clear that the biochemical change is the primary result of the mutation and that the loss of virulence for the mouse follows as a consequence.

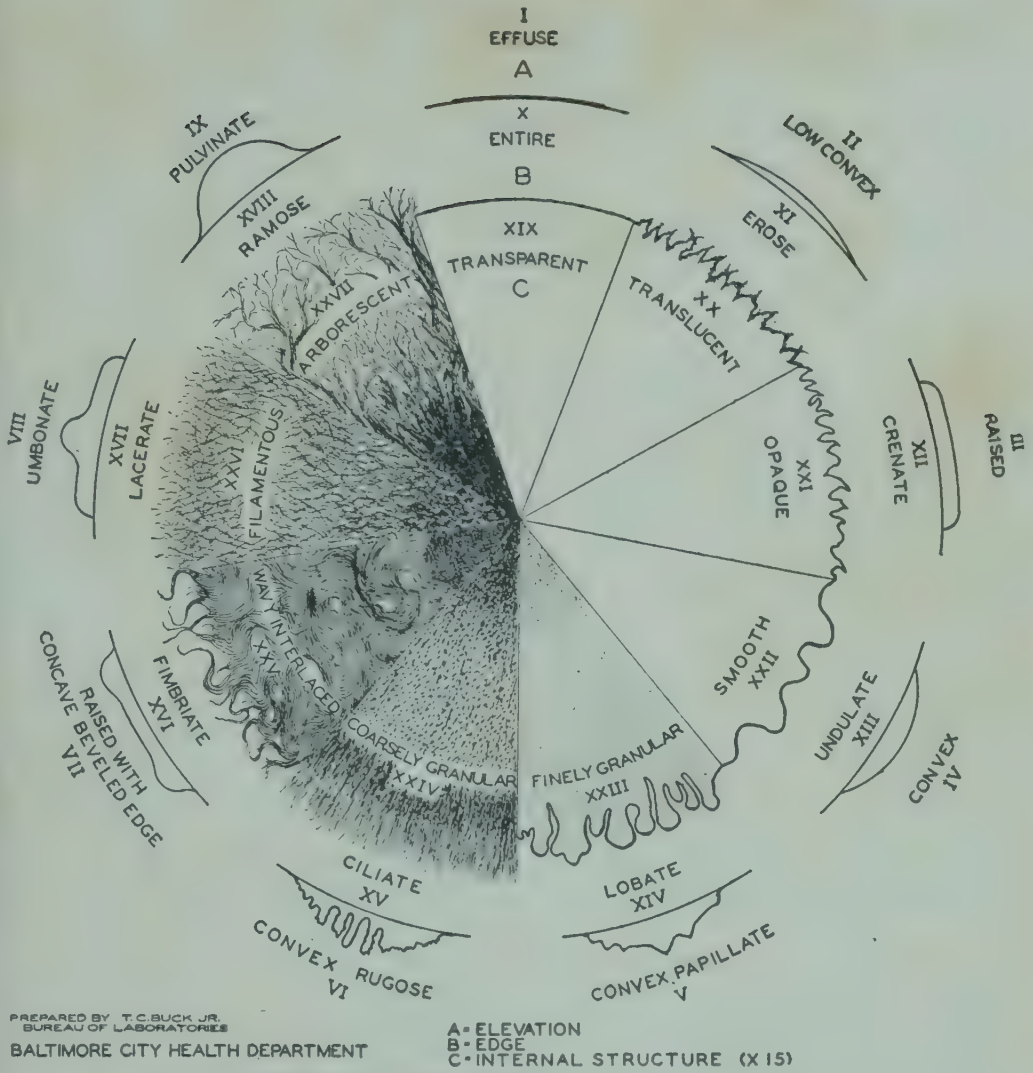


FIG. 72. Bacterial colony formations, a schematic representation of types of bacterial colonies. While the above pictorial representation of a nomenclature suggested for describing the different kinds of colonies has not been generally adopted, it has the merit of greater definiteness in meaning than the more commonly used device of referring to colonies as S, R, SR, etc. Depending on the organism studied and the investigator, the colonial types illustrated in the right half have been all called smooth and those on the left rough, with no clear line of demarcation between intermediate (RS) types and rough and smooth. Use of the scheme would simplify descriptions of colonies by making it possible to use numbers only to designate the type of colony observed. Four numbers will adequately describe any colony, as can be seen by reference to the chart. For example, if a colony is recorded as II, XIII, XXI and XXIII, the colony would be one with a low convex elevation, an undulate edge, and an opaque, finely granular internal structure.

A little thought leads to the conclusion that changes in the character of the surface properties of a bacterial strain must have many consequences. For example, if an encapsulated strain of pneumococcus loses the capacity to produce a capsule the colonial appearance will no longer be mucoid. At

TABLE 37

*Characteristics most commonly associated with "normal" and "mutant" types of culture*

S, NORMAL TYPE	R, MUTANT TYPE
Homogeneous clouding in broth	Agglutinative growth in broth
Normal suspension in 0.8% salt soln.	Sedimentary suspension in 0.8% salt soln.
Fair, conservative growth on agar	Often expansive growth on agar
Colonies smooth, regular, convex	Colonies rough, irregular, flat
May generate secondary colonies	Seldom generates secondary colonies
Agar growth soft, opaque	Agar growth harder, translucent
Agar growth fluorescent	Agar growth seldom so fluorescent
Agar growth pyocyanogenic	Agar growth nonpyocyanogenic
In motile species, active	In motile species, nonmotile
Possessing distinct capsules	Noncapsulated
Biochemically more active	Biochemically less active
Carries double antigen (S and O)	Often pure R; may have some O or S
Generates the "specific soluble substances"	Lacks the "specific soluble substances"
Flocculogranular precipitate in serum	Flocculent precipitate only in immune serum
If a pathogen—virulent (or toxic)	Slightly or nonvirulent (nontoxic)
More common in active disease	More common in carriers and convalescents
More common in acute infections	More common in chronic infections
Sensitive to aging	More resistant to aging
Sensitive to bacteriophage	Less sensitive to bacteriophage
Represented by freelifing forms	Product of adaptations
Cells of "normal" morphology	Tendency to short rods and cocci
Transformed to O or R in S immune serum	Not transformed in S immune serum
Not transformed in R immune serum	Transformed to S in R immune serum
Relatively resistant to phagocytosis	Susceptible to phagocytosis

(From Hadley, 1927.)

the same time the organism will have changed in antigenicity because the capsule is composed of specific antigenic material. Since the presence of a capsule adversely affects the capacity of phagocytic cells to engulf these cocci, the unencapsulated form will be more easily phagocytosed than the parent strain and on injection into mice will be observed to have a reduced virulence. Furthermore, since the capsular material is a markedly hydrophilic substance it is not surprising that the unencapsulated dissociated



organisms may be more readily agglutinated by salts and acid and may even show a tendency to grow in clumps in broth media. The loss of the capsule has a biochemical origin. The inability to produce a high molecular weight carbohydrate containing nitrogen may well be the end result of some metabolic change distantly removed from the final steps of capsule formation. It is not surprising that the biochemical activity of the dissociant could vary in a number of ways from encapsulated types.

The evidence for the causal relationship of gene mutation to dissociation is increasingly impressive. At the least the analogy to gene mutation provides the most productive working hypothesis for studying problems of dissociation. A most significant finding in support of such an analogy is the occurrence of independent changes of characters. There is an absence of orderly succession in the appearance of dissociant phases. Moreover, reversion takes place and can occur in the very environment in which the original dissociants appeared. Finally there is evidence against the direct correlation of the type of dissociation produced with specific environments. All these facts resemble true gene mutation which is spontaneous and random.

Now while it is true that the observation and isolation of dissociants from a parent culture is favored by manipulating culture techniques so as to extend the period of multiplication by aging the cultures or by growing bacteria in unfavorable environments such as media containing lithium chloride, these facts do not militate against the notion of gene mutation. Actually they suggest selection of spontaneously occurring mutants since extended periods of multiplication would increase the opportunity for the actual occurrence of mutation. Unfavorable environments for the parent type can selectively favor the survival of mutants unlike the parent type. In this connection a most significant contribution has been made by finding that as a culture ages metabolic products may accumulate which selectively inhibit the growth of the parent strain and permit the overgrowth of the culture by mutants. With *Brucella abortus*, alanine has been shown to accumulate in a smooth phase culture and by its presence to create an environment more favorable for the growth of dissociants of the rough type.

From natural sources the smooth phases (or M in the case of capsulated pathogens) of bacterial species are those isolated most frequently, and this predominating presence of the smooth types in natural environments requires explanation. Doubtless the cause must lie in a greater competitive survival value of the properties of the smooth phase in nature. How natural selection favors the predominance of these properties is a matter for speculation.

It is suggestive that the greatest biochemical activity of bacterial species is associated with the smooth phases. In this connection one view of the

direction of organic evolution has been that it proceeds to maximize the total flow of energy through the whole biological world. In keeping with this tendency the natural environment would be occupied by those types which attack natural substrates at the most rapid rates and which are capable of acting on the greatest number of different available substrates and thus are provided with a competitive advantage under the circumstances of mixed culture in natural environments. Consequently smooth forms, as the more energetic types, would tend to constitute the wild populations of bacteria.

### *Mutation Rate and Change in the Character of a Population*

If a change in the composition of a population is noted, is it the result of the accumulation of spontaneously occurring mutants, or is it the result of selection which favors the growth of some more successful variant? From the mutation rate it is possible to calculate the accumulation of a mutant in a population and to arrive at an answer to the question.

If  $u$  is the mutation rate expressed as mutations per bacterium per division cycle and  $q$  the fraction of mutants present in a population at any given time, then  $G$ , the number of generations required to shift the population to include  $q$  fraction of mutants after the first mutant appears, may be found by substitution in the following equation:

$$G = -\frac{1}{u} \ln q \quad (1)$$

This equation was derived in a study with *Phytomonas stewartii*, and the data have general significance. With a mutation rate of  $1.81 \times 10^{-5}$  it was found that 38,200 generations or approximately six years of growth and multiplication of this bacterial organism would be required to shift the composition of a population so as to include 50 per cent of the mutant type. Obviously mutation rates alone cannot account for the changes in the composition of bacterial populations which are actually observed within the growth cycle of a single culture. Selective forces must be operating either to suppress the metabolic and multiplication rates of the parent type or to favor those of the mutant. Mutation supplies the materials but it is the play of selective forces which modifies the character of populations.

The genetic composition of populations will be the sum of both the forward and reverse mutation rates and the operation of selective forces. A simple proportion aptly summarizes the situation for a population in a state of equilibrium:

$$\frac{M}{N} = \frac{f}{b} \quad (2)$$

where  $M$  is the number of mutant bacteria,  $N$  the number of the parent type of bacteria,  $f$  the forward mutation rate, and  $b$  the back mutation rate. The operation of selective forces may be taken into consideration. Let  $s$  represent a selection coefficient, that is the difference in viability or multiplication rates of the parent and mutant types, then at equilibrium and ignoring the influence of back mutation:

$$\frac{M}{N} = \frac{f}{s - f} \quad (3)$$

### *Induced Versus the Spontaneous Origin of Variants*

In order to detect bacterial mutation it is often convenient in practice, and sometimes necessary, to employ a selective environment for the separation and consequent detection of a variant. Of course this procedure poses a problem of interpretation. Is it certain that the selected types originate by spontaneous mutation or is their origin actually induced by the selective environment?

If one is dealing with a character that does not require the use of a selective medium for its detection, an answer to this question is directly forthcoming though it may require tedious work with a great number of colonies or individual cultures. For example, if a search were being made for the organisms fermenting lactose that originate from a non-lactose fermentor, the populations of related clones could be plated out on a lactose-agar medium containing an acid-base indicator. The reaction of the medium surrounding the individual colonies would reveal whether lactose was being fermented or not. In this case since both the lactose fermentors and non-fermentors derive from a common ancestor and are developing in the presence of lactose it is obvious that the presence of the lactose cannot be responsible by direct induction for the appearance of any lactose fermentors. In order to avoid growth in the presence of the sugar, tests for lactose fermentation could be done with resting cell suspensions derived from numerous cultures originating from a common ancestor and grown in a lactose-free medium.

There are other properties of variants such as the capacity to resist chemical poisons and lysis by bacteriophage which by their nature require for their detection the use of a selective environment for isolation of the organisms and for which, therefore, a specific inducing role for the selective environment cannot be immediately excluded. Two general procedures are available to determine the role of a selective environment in the origin of such variants.

In one method small and equal quantities of non-resistant organisms from a clone are spread on three series of plates. One series contains the



toxic material against which resistance is to be tested and is incubated immediately after inoculation. These plates serve as a control to indicate the absence of resistant types in the original inoculum. The second and third series of inoculated plates contain medium without added toxic test material and are incubated just long enough to permit multiplication and the formation of microcolonies. After this preliminary incubation the toxic test material is added. The second series is immediately reincubated. In the third series, however, a sterile platinum loop or glass rod is rubbed over the surface of the plates in order to spread the growths which have occurred in the microcolonies, and the plates are then reincubated.

Differences in the number of visible colonies developing on these test series of plates provide the data which indicate the role of the toxic agent in the development of resistant forms. If the number of colonies on the third series is the greater it means that resistant individuals developed during the growth of the microcolonies but prior to the addition of the selective toxic test material. Thus the spreading of microcolonies in which such individuals appear and multiply before the addition of the selective agent would result in an increased number of colonies of resistant organisms in the selective medium. An increase in the number of resistant individuals by multiplication in a medium free of the selective agent would be predicted on the assumption that resistance develops by spontaneous mutation. In any case resistance cannot be induced by the selective agent when the agent is absent, this being the self-evident principle on which the interpretation of the difference in number of visible colonies on the spread and unspread series of plates is based.

The role of a selective environment in the appearance of variants may also be analyzed by means of a *fluctuation test*. In this procedure a series of tubes of nutrient medium free of the selective agent is inoculated with a small quantity of organisms from a clone of organisms non-resistant to the toxic test material. Growth is permitted to proceed beyond the exponential growth phase. Then an equal sample from each tube is spread on separate plates of medium containing the toxic test material, and from one of the tubes a large series of plates is inoculated rather than a single plate. Comparison of the variation in the number of resistant colonies developing on the plates inoculated from the different tubes with that of the series inoculated from the single tube provides the answer sought. A greater variance in the number of colonies on the plates from the series of tubes indicates spontaneous mutation as the cause of the origin of resistant variants. This conclusion is drawn because while the mutation rate for a given gene is characteristic the actual occurrence of the first mutant in each tube of a series inoculated with a genotypically homogeneous population is completely random with respect to time. Therefore, variation in the total

number of mutants and their progeny in the individual tubes of such a series will be large by the time the growth cycle is completed. On the other hand, if resistance is an induced and adaptive response to a selective agent, a series of plates containing the selective agent with each plate inoculated with the same number of organisms from one original clone should show a minimum variation in the number of resistant colonies which appear among the plates. The variance in the number of resistant organisms should be the same in both series of plates inoculated in the fluctuation test since

## EXPERIMENT TO TEST FOR GENETIC BASIS OF RESISTANCE TO ANTIBIOTICS

METHOD: LURIA AND DELBRÜCK, GENETICS, 1948, 28:491  
DATA: DEMEREC, J. BACT., 1948, 56: 63

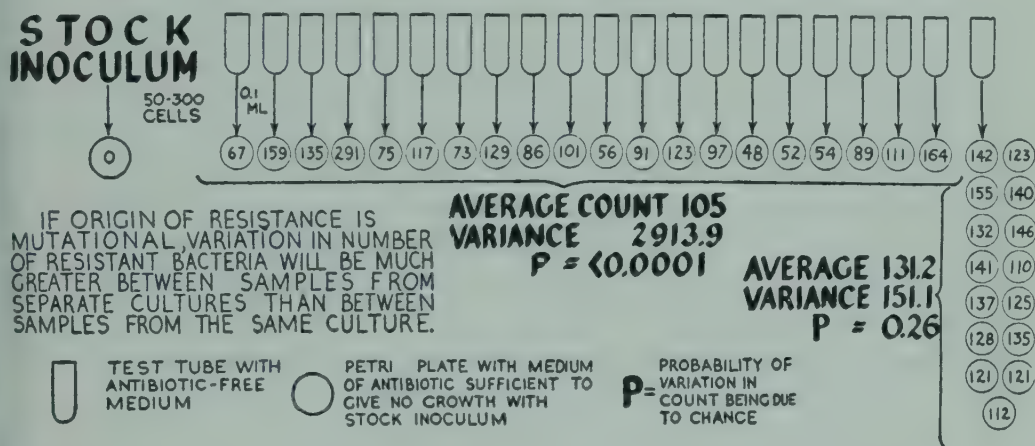


FIG. 73. Set-up and data of a fluctuation test which indicates that the origin of resistance to penicillin is spontaneous and independent of the presence of penicillin. In the case cited *Staphylococcus aureus* was the species studied.

the opportunity for an adaptive response to the selective agent in each plate is presumably the same. A diagrammatic representation of an actual experience with the fluctuation test is shown in Figure 73.

### Development of Resistance to Poisons

By a process called *training*, that is, repeated exposure of survivors to increasing toxic concentrations of a poison, it is possible to isolate bacteria of astonishingly increased resistance to a toxic material relative to the resistance of a parent culture. A heritable change is indicated since the organisms may retain their increased resistance in spite of repeated subcultures through media free of the drug. Most of the organisms from a normal culture will multiply when seeded into a slightly toxic concentration of



a poison, such as an antibiotic. The progeny of these organisms prove to possess a higher resistance to the antibiotic than the parent culture, an observation indicating a heritable mass transformation of resistance within a population. The degree of increased resistance obtained by training is most often found to be related to the concentration of the poison at which the organisms have grown out. These facts and the additional one that a spectrum of strains of all the possible resistances may be isolated have raised the question as to whether it is not more logical to attribute the increase in resistance to an adaptive response to the toxic factor rather than to selective forces acting on a large number of separate and step-wise gene mutations. Inasmuch as all the pertinent observations do not point to the same conclusion a clear cut decision between these possibilities has not been rendered.

If a colony growing on a plate of medium containing a toxic substance had its origin from a resistant mutant then, except for back mutation to a state of reduced resistance, the great majority of the organisms in the colony should be of the resistant type. With regard to resistance to streptomycin this expectation is not always realized as the data in Table 38 illustrate. Lacking independent evidence it is difficult to explain away such data by assuming an unusually high back mutation rate and the operation of selective forces in favor of non-resistant mutants during the manipulations attending the second transfer of populations from an originally resistant colony into media containing antibiotic. It is equally true that these data do not suggest an uncomplicated process of adaptation.

Nonetheless there are important observations which do suggest mutation as a cause of increased resistance to poisons:

- 1) In populations without a previous history of exposure to the drug a small number of resistant individuals is always found (Table 39). The numbers found are in agreement with the assumption that mutation is a rare event.

- 2) Training is not always necessary to achieve high levels of resistance starting with strains of low initial resistance. In some cases single step changes in resistance have been observed which are equal to the total increase resulting from training.

- 3) Results of the fluctuation test favor the concept of the mutational origin of resistance to antibiotics (fig. 73).

- 4) With strain K-12 of *Escherichia coli* resistance to chloromycetin may be raised in two-fold and lower steps starting with concentrations of 5-10 micrograms of drug to 1,280 micrograms per milliliter. Matings between resistant and non-resistant strains yield gene linkage data which are consistent with the possible existence of many genetic loci for resistance with a cumulative action.



It has been suggested that the origin of resistance may be both by mutation and by adaptation, the later being the more likely event when one is dealing with the slight increases in resistance developed by organisms

TABLE 38

*The resistance of individuals in a resistant colony of Aerobacter aerogenes from plates containing streptomycin*

COLONY NO.	STREPTOMYCIN IN PLATE	COUNTS ON SECOND PASSAGE	
		Control plate	Streptomycin plate
	units/ml	No. of organisms/ml	
1	2.5	$90 \times 10^6$	$70 \times 10^6$
2	1.3	$20 \times 10^6$	$1 \times 10^6$
3	2.3	$10 \times 10^6$	$0.7 \times 10^6$
5	3.3	$300 \times 10^6$	25
9	3.5	$40 \times 10^6$	25
11	4.4	$7 \times 10^6$	c. 13
12	4.4	$20 \times 10^6$	$1 \times 10^3$
13	8.0	$10 \times 10^6$	0
14	16.0	$2 \times 10^6$	0

If gene mutation to a state of increased resistance were the cause of the ability of an organism to form a colony in the presence of an antibiotic, the population of the colony should consist of resistant progeny capable of growth at the same concentration of antibiotic as the parent organism. For all but colony number 1 the above data do not confirm this expectation.

(From Barer, 1951.)

TABLE 39

*Number of resistant forms obtained from normal populations*

ORGANISM	NUMBER OF COLONIES			
	In plain agar	In NaCl agar	In HgCl <sub>2</sub> agar	In Cu <sub>2</sub> SO <sub>4</sub> agar
<i>Salmonella pullorum</i> .....	800,000,000	15	21	48
<i>Salmonella schotmülleri</i> .....	480,000,000	120	16	29
<i>Eberthella typhosa</i> .....	440,000,000	61	32	30

(From Severens and Tanner, 1945.)

exposed to slightly toxic concentrations of poisons. No doubt this compromise point of view is attractive and will be the subject of further study. However, conclusive proof of any instance of an adaptive and heritable mechanism of resistance would raise the important question of how such an adaptation is engineered by the organism. Certainly the question of a cytoplasmic as opposed to a nuclear mechanism of inheritance would be

advanced. In such an event the existence of strain K-12 might prove of immense value, if an example worth investigating were found in this organism, because the known methods of testing for cytoplasmic inheritance depend upon the use of organisms with sexual modes of reproduction (Caspari, 1948).

#### GENE RECOMBINATION AND SEGREGATION

Evidences for sexual reproduction in bacteria have long been sought, but for the most part cytological examination of preparations for the detection of fusion of bacteria has been negative. While claims do exist for the direct observation of the sexual union of bacteria in stained preparations, the fusion, if it actually takes place, has never been followed in its details as a process occurring in living cells. Nor have the conditions of observations of stained systems been such as to permit repeated demonstrations at will by different investigators. The chance juxtaposition of bacteria or the agglutination of living individual organisms for reasons more concerned with extraneous variations in the physical chemistry of their surfaces rather than with sex have not been eliminated as the more probable explanations of those cases where direct microscopic evidences of fusion have been reported. Definitive genetic evidence of sexual processes obtained by the isolation of bacteria with hybrid characteristics from the same preparations in which visual evidences of fusion have been claimed has not been forthcoming.

The more successful approach has been a genetic one, a search for hybrid progeny of unlike parents. However, strain K-12 of *Escherichia coli* is the only bacterial organism known which exhibits clear and uncontested evidence of a sexual process.

If two clones of a bacterial species which differ in some identifiable way were to be mixed and sexual reproduction occurred, organisms with hybrid characteristics should appear in the mixed culture. However, if the character differences of the parents involved only one allelic gene pair, mutation might occur which could result in progeny simulating the hybrid character expected from the result of sexual union. Therefore, it has been desirable to work with parent types which differ in two or more independent characteristics. The odds against a mutation for two or more independent characteristics in single individuals are so high as to be precluded as an explanation if a significant number of progeny of hybrid character were isolated from a mixture of unlike parents. The occurrence of just such hybrids has been described in strain K-12 of *Escherichia coli*. Mixtures of more than two types of parent strains apparently do not result in progeny derived from other than pairs of parents.

A few other strains of *Escherichia coli* have also been isolated that show



evidence of undergoing gene recombination. K-12 is a wild strain. The isolation of different strains from natural sources may actually be repeated but unidentifiable isolations of K-12. This argument would not be tenable if other wild strains of *Escherichia coli* which show evidence of sexual reproduction among themselves could be shown not to mate with K-12. On the other hand matings between other wild strains and K-12 would be neither pro nor con in their bearing on the question of how widespread sexual processes are among the strains of wild types of *Escherichia coli*.

The reason for the inability to demonstrate by the genetic method sexual reproduction of bacteria other than *Escherichia coli* is unexplained. It may be that the phenomenon more generally involves heterothallism, and bacteriologists may not have been so fortunate as to have mixed by chance the proper mating types of strains. In addition, in organisms other than K-12 the range of environmental conditions necessary for sexual reproduction may be so narrow as to have not been met in experimental work to date.

In the original demonstration of sexual reproduction of *Escherichia coli*, mutants of K-12 were obtained by irradiation which, unlike the parent type, were unable to synthesize various growth factors. While mixtures of such variants did not result in the appearance of progeny with the deficiencies of both parents, there did appear prototrophic organisms able to synthesize all of the growth factors missing from the biochemical makeup of both parents. While the yields of these prototrophic types were so small as to account for the inability to note sexual union by cytological means, they were sufficiently numerous to exclude gene mutation and reversion as the cause of their origin.

In the light of the data available the following process of sexual reproduction in K-12 may be drawn. The parent organisms appear to be haploid. Sexual union results in the formation of a diploid bacterium. As a rule the diploid organism does not multiply as a diploid. Rather it undergoes a meiotic reduction division resulting in haploid progeny. In what must correspond to the pachytene stage of meiosis in other organisms, crossing over between homologous chromosomes appears to take place thus accounting for the origin of haploid progeny with hybrid characteristics. A presumed and idealized picture of these events is illustrated in Figure 74.

Infrequently heterozygous diploids capable of multiplying for some time as diploids before they undergo a reduction division to yield haploid progeny are isolated from the mixture of parent types. Using these organisms it has been possible to prove by single cell isolation of progeny the coincident presence of the genetic factors of two parent types within an individual bacterial organism (fig. 75).

The study of the process of gene recombination and segregation has also led to the conclusion that the strain of *Escherichia coli* involved pos-



sesses a single chromosome. A preliminary picture of the spatial and linear distribution of some genes within this chromosome has also been mapped. It will be recalled that the closer two genes are on a single chromosome the

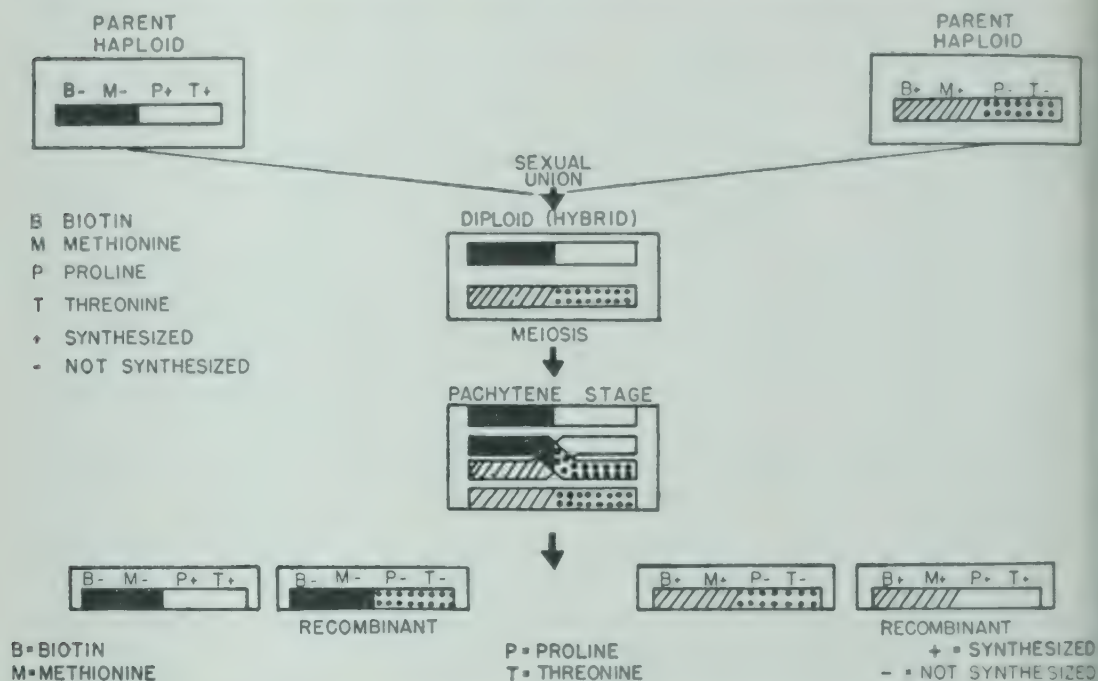


FIG. 74. An idealized version of gene segregation and recombination in *Escherichia coli*.

The imaginative contribution of the original genetic studies of sexual reproduction of bacteria by Tatum and Lederberg which were responsible for the successful demonstration of the process was the use of selective media or environmental conditions for screening out the recombinants from populations largely composed of parent types.

Conditions were chosen which did not permit the growth of parent types but did permit the growth of the recombinant types. In the case illustrated the use of a medium deficient in biotin, methionine, proline, and threonine effectively selects the prototrophic recombinant. The separation of the completely deficient type from mixtures of the parent types is a more difficult technical problem. For this purpose, as well as for the purpose of estimating the frequency of crossing over, single cell isolations of progeny from diploids should be a direct and certain, even if laborious, method of procedure. In actual fact the completely deficient types of recombinant have not been isolated and one suspects that more than a technical difficulty is involved in this negative result (see Figure 75 for further discussion).

more likely are they to travel together when crossing over takes place during meiosis. The deduced frequencies of crossing over involving linked genes provide the data for the chromosome map of *Escherichia coli* (Lederberg, 1947).

The existence of the phenomenon of gene recombination and segregation

in a bacterial species presents many opportunities for the study of the nature of the genetic mechanism and certain problems of biochemistry. As a result, the phenomenon holds great interest for the geneticist and biochemist

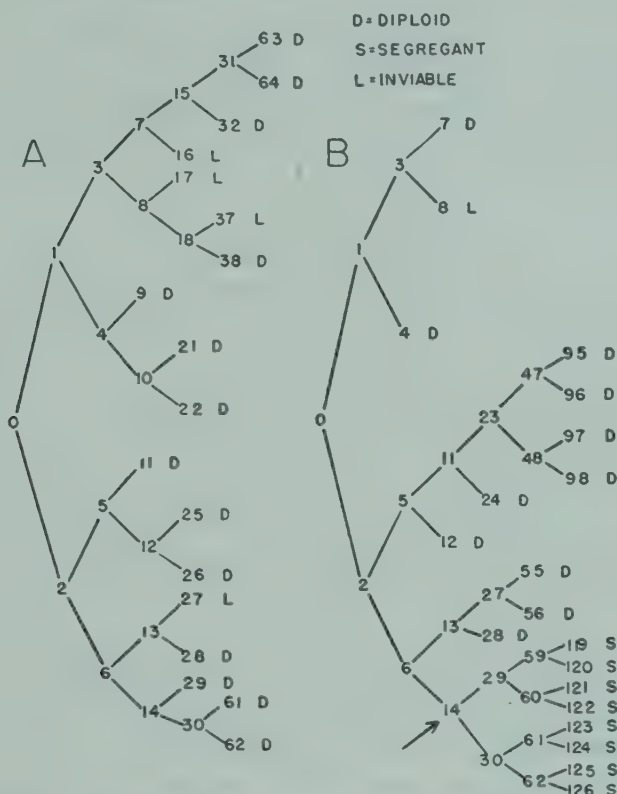


FIG. 75. Single-cell pedigrees of diploid *Escherichia coli* (H-226). A: Pedigree in which no segregation has occurred. B: A pedigree involving segregation. Cell number 14 (arrow) represents the point at which segregation occurred. The high incidence of lethals appears to be characteristic of pedigrees of diploid cells.

(From Zelle and Lederberg, 1951)

The typical picture of meiosis illustrated in Figure 74 probably does not occur in the case of sexual reproduction by *Escherichia coli*. If it did certain predictable ratios of various kinds of haploid progeny from segregation diploids should be isolated. Zelle in a personal communication has summarized the actual picture found: "From any particular segregating diploid cell one obtains one haploid type with the sibling cell being either inviable or else a diploid. We have never obtained the two complementary types of segregants as one would expect if the process were an uncomplicated meiotic one. From other evidence one suspects that the diploid was heterozygous for a deficiency as well as the various genes whose segregation we study. If this is true then one would not expect to obtain the complementary types. It is interesting however that from a large number of segregants under these conditions in which there is no opportunity for differential growth of the segregants, the expected proportion was quite closely approximated. Incidentally the observation of an individual cell giving rise to two cells, one of which is a haploid segregant and the other a segregating diploid, is critical proof that at least this strain of *Escherichia coli* is multinucleate."

as well as the bacteriologist. Yet the great interest in the subject should not obscure its probable unimportance in explaining the vast majority of the variations of bacteria observed in nature and the laboratory. Developmental variation and gene mutation remain the overwhelming causes of the variations observed. If sexual reproduction is present in many species, it probably occurs at a very low rate relative to asexual reproduction and takes place for the most part within pure lines where it cannot lead to variation. When differences between parents do not exist sexual reproduction cannot result in differences.

#### TRANSFORMATION

Transformation has been defined as a "hereditary alteration in a susceptible cell resulting from the acquisition from its environment, by other than sexual means, of a genetically active unit directing the inheritable change". In fact the genetically active unit directing the transformation is a desoxypentose nucleic acid. In a suitable bacterium this material induces a change to a characteristic associated with the organism from which it was derived. The affected characteristics of the transformed organism which have been investigated largely involved the antigenic specificity of cellular components particularly those making up the surface cellular layers. The fermentative and other physiological properties of the organisms have not been intensively studied in transforming experiments. From the transformed organism and its progeny the transforming substance is recoverable in greater quantities than was required for the original transformation. Hence the transforming substance seems to autocatalyze its synthesis within the transformed organism.

Successful transformation experiments involve the satisfaction of three requirements:

- 1) An organism capable of being transformed, spoken of as *competent*, must be present. Transformation involves intraspecies changes. In pneumococcus competent cocci are rough dissociant or unencapsulated organisms which are so transformed as to synthesize the capsular polysaccharide of the type from which the transforming substance is derived.

- 2) Transforming substance must be provided during a stage of active growth and sometime after the inoculation of competent bacilli into a nutrient medium. Lysing cultures, sterile culture filtrates or purified desoxypentose nucleic acid may be used.

- 3) Environmental conditions must be provided under which the transforming substance and competent cells will interact. In the pneumococcus this includes addition of specific antibody or other substances leading to the agglutination of competent cells, an unknown non-dialyzable component of serum albumin, and pyrophosphate. With other organisms such



as *Hemophilus influenzae* the environmental conditions for transformation do not seem to be as exacting as for pneumococci.

Successful transformation is limited to a small percentage ( $<0.001\%$ ) of treated organisms. It is not known whether this low rate is a reflection of exacting external environmental requirements or a fleeting acceptable physiological state of the host cells for the transforming substance.

The effect of the addition of transforming substance may be entirely prevented by the use of purified desoxyribonuclease. This has been shown by noting the effect on the transformation process of the addition of desoxyribonuclease to a growing culture of competent cells at varying time intervals. The growth of the competent cells is required for the fixation or uptake of the transforming principle. After an initial period of sensitivity during growth, the system becomes resistant to the interfering action of the enzyme.

The problem of the biological nature of the transforming desoxypentose-nucleic acid is an intriguing one. The substance cannot be an intact portion of a chromosome since typical chromosome structure includes protein components. If it is the desoxypentosenucleic acid fraction of a chromosome, is it representative of the material that makes up a single gene?

Associated with the problem of the biological nature of transforming substance is the question of its place in the transformed organism. If it is normally a part of the hereditary apparatus of the parent strain does it also enter into any chromosome structure which may be present in the transformed organism? If so does it displace an allelic gene which normally determines the characteristics in the transformed cell? Or does  $S \rightarrow R$  dissociation result in the loss of a gene which can be replaced by the transforming desoxypentosenucleic acid? Is this why R phase organisms are competent when parent S phase organisms are not? But if  $S \rightarrow R$  dissociation involves the loss of a gene how can the gene be reacquired in  $R \rightarrow S$  mutation? A solution to these questions touches on many fundamental problems of the morphological organization of genetic factors at the sub-microscopic level. It is no wonder that the work on bacterial transformation has attracted so much attention.

### LIFE CYCLES OF BACTERIA

The variations noted with bacteria, especially those related to dissociation, have occasionally been interpreted as expressions of life cycles. These reports have consisted of data obtained in isolated observations reconstructed to fit into some presumably logical cycle of events. As a result it has not been possible to repeat observations at will. Nor have the original investigators succeeded in communicating the conditions necessary for the appearance of successive phases of development in so precise a

manner as to permit confirmatory observations by other and disinterested bacteriologists. The hypotheses of life cycles have had therefore a rather checkered career and have not received general acceptance. In spite of the unsatisfactory state of the subject it will be worthwhile discussing critically the problem of bacterial life cycles. Our purpose will be to outline the criteria by which the merits of publications of life cycles may be judged rather than to discuss the acceptability of particular studies which have attracted attention. In addition the search for an answer to the question of life cycles in bacteria illustrates problems of scientific methodology of general import in attempts at the scientific explanation of phenomena.

Where life cycles do occur they are of fundamental importance in understanding biological phenomena. Consequently the problem should not be abandoned on the basis of negative evidence without a thorough exploration of the possibilities. The criteria which may be used for validating any hypothesized life cycle are several. An orderly succession of phases should be observed. The completion of one full turn in the cycle should be possible irrespective of the phase at which observations are begun. If certain environmental conditions are necessary for the expression of a phase these conditions should be specified. Associated characters of a phase should not change independently nor occur at random among the other phases of the cycle. Hypotheses of complex life cycles in bacteria have not met these criteria. The deficiencies encountered may be exemplified by considering briefly the claims for the existence of filterable forms in bacterial life cycles.

The presence of a filterable form has usually been based on the discovery of visible evidence of growth in media which have been inoculated with culture filtrates. Frequently the observations have required an extended period of incubation of the media, and the growths observed often have been non-persistent and easily lost on serial transfer. The forms developing on the media inoculated with filtrate have not resembled the parent type, nor has it been possible to get reversion in appearance to the parent type. These are serious deficiencies in attempting to relate a filterable form to a phase in a bacterial life cycle. On the basis of the evidence it may be equally logical to propose alternative hypotheses.

The inability to maintain forms originating from culture filtrates might be because they are non-living in nature. They might be the slightly soluble products of reactions catalyzed by enzymes present in the filtrate and involving substrates present in the media employed. Subculture will dilute out these enzymes and thus account for the non-persistence of the "filterable form". This possibility, often ignored, must be eliminated before a non-persistent "growth" is assumed to be genuine because the extracellular production of high molecular weight substances or slime layer polysac-



charides and polypeptide in the absence of living cells is known to occur. In addition, cultures of certain bacteria in solid media tend to form amorphous precipitates of inorganic salts or *calculi*, which are not readily distinguishable by their appearance alone from pin-point and petite colonies. The possible validity of this explanation in particular cases is suggested by the indefinite or amorphous morphology ascribed to "growths" from filtrates. Certainly the *synplasms*, plasmatic masses of amorphous appearance attributed to the promiscuous intermingling and dissolution of vegetative cells in a sort of mélange of sexual fusions, may well have their origin in extracellular enzymic activity and thus have no significance for the life cycles postulated by the bolder pleomorphists.

To admit that the growths from culture filtrates are genuinely living organisms still leaves the possibility of their origin in contamination unless this pitfall can be definitively excluded. This necessary exclusion may present a more formidable problem than is generally appreciated. Contamination of culture filtrates and of the media into which they are introduced can be a very subtle process. In apparently sterile media incubated for long periods of time slowly growing organisms, including imperfectly characterized diphtheroids, often make their appearance. These are generally missed in routine bacteriological work employing incubation periods of the order of 48-72 hours. Nonetheless, they have presented problems for persons testing for sterility of media over extended periods of incubation. The reality of the problem is attested to by the experience of those pharmaceutical manufacturers who have conscientiously tested their chemical and biological products for sterility by incubating the inoculated test media for long periods. Invariably the richer the nutrient medium and the longer the period of incubation employed the greater the number of negative tests obtained for sterility.

In general, the visible manifestations of filterable forms in bacterial filtrates have been studied in petri plates. The air to which these plates are exposed during pouring, incubation, and handling during periodic observations may be the ultimate source and the opportunity for unappreciated contamination. Moreover, the phenomenon of dormancy may also play a role. The reports of filterable forms are based on the use of non-synthetic media and commonly include serum products as an enrichment. Such media are known to contain numerous and as yet incompletely defined materials favoring the dormancy of the few organisms which might penetrate a bacterial filter or of the few contaminants unconsciously introduced during the experimental manipulations of the filtrate. Such materials may thus be responsible for the extended periods of observation required for evidence of growth from bacterial filtrates. In any case these are examples of the kinds of alternatives which must be rigidly excluded before a filter-



able form can be accepted as part of a life cycle from the kinds of data which have been presented in the past.

When the label "life cycle" is attached to a series of cyclic appearances within a species what is the basic nature of the biological phenomenon being described? In animals one may observe a cycle in appearances. An adult moth lays an egg, the egg yields a caterpillar, the caterpillar soon spins a cocoon about itself wherein it rests as a pupa until the coming of spring when it emerges in the shape of its original parent. Such a cycle of events, marked changes in form during post embryonic development, is *metamorphosis*. Might bacterial life cycles be in the nature of a metamorphosis? It hardly seems likely. Metamorphosis is a cycle in the appearance of an *individual* harmonized in relation to cyclic changes or to the seasons of its natural environment. Within its life span as an individual organism a bacterium is not exposed to a natural environment varying regularly in cyclic fashion. A bacterial population during its growth cycle does show characteristic changes in average morphology and physiology in a continuously changing environment. While the temptation may exist to apply the term metamorphosis to these changes, which have been called cytomorphosis by bacteriologists, the phenomenon is still not in strict analogy with the metamorphosis of animal forms.

Another kind of life cycle is the alternation of generations exhibited by sexually reproducing plants. The alternation is fundamentally a reflection of differences in nuclear organization. A haploid or gametophyte stage follows a diploid or sporophyte stage which in turn can yield the gametophyte stage to complete the cycle. It is possible that the bacterial endospore represents one phase of such a life cycle in the spore-forming species. This, however, will remain a merely speculative hypothesis until exact knowledge develops of what, if any, nuclear changes occur in the processes of sporulation and spore germination. In the strain K-12 of *Escherichia coli* there is justification for the use of the phrase life cycle to mean an alternation of generations. But at the present time in view of our deficiencies of cytogenetic knowledge of the bacterial apparatus for transmitting hereditary factors and the lack of acceptable genetic evidence for a sexual process in other bacteria, this type of life cycle can hardly be claimed to exist among bacteria other than *Escherichia coli*.

If the transfer of bacteria into various media results in the regular appearance of different variations are these a reflection of a life cycle? They are not if the characteristic appearances in the media are the result of selection of different mutants by the media. True life cycles should be demonstrable within a pure line.

Within the confines of laboratory cultivation, observed cyclic variations in a pure line may be due to developmental variations as an immediate

response to environmental changes. These hardly deserve status as life cycles unless it can be shown that the natural environment of the organisms also provides equivalent cyclic changes. This type of variation, even if it could be shown to occur in natural populations, would not have the genetic significance of a true alternation of generations. It would be equivalent to applying the term life cycle to the fluctuations in the body weight of a species of trout in a lake with the seasons of the year which occur in response to the variations in the quantity of food available. This use hardly seems an appropriate one for the term life cycle and emphatically is not the meaning assigned to the term by biologists.

*L forms* of bacteria have been described which resemble the pleuropneumonia organisms in morphological appearance. These forms are non-rigid, fragile, and in size may be near the limits of the resolving power of the light microscope. They may be filamentous or branching and reproduce by segmentation of a filament. Bacteria may balloon or swell in which condition they are known as *large bodies*. There are two points of view regarding the nature of such forms, namely, that they are a normal phase in a life cycle and, alternatively, mere fluctuation or pathology in response to harmful environmental influences. The fact that *L forms* can be induced in many species by exposure of sensitive strains to antibiotics and other poisons inclines one toward the latter hypothesis. If the presence of toxic materials affects growth rates in a quantitatively different manner than fission rates or interferes with the synthesis of cell wall components responsible for the mechanical rigidity of the bacterium, it is not surprising that morphology will vary. In any case the proponents of the life cycle hypothesis of the origin of *L forms* have not shown in what manner their hypothesis is more logical than an alternative hypothesis.

#### BACTERIOPHAGE

Viruses specifically parasitizing bacteria are called *bacteriophages* or simply *phages*, names derived from the dramatic ability of these viruses to cause lysis of infected bacteria. The lysis is indicated by the clearing of turbidity in broth cultures or by the appearance of clear areas or *plaques* in the film of growth of bacteria on the surface of an agar plate. By correlation with direct electron microscope counts of phage particles it has been definitively established that a single phage can infect and cause lysis of the individual bacterium and thus give rise to a clone of phage able to lyse the bacteria in a broth culture or form a plaque on an agar culture. Consequently the number of phage particles in a preparation is estimated by dilution to the point where lysis no longer occurs in a broth culture of susceptible bacteria, or by counting the plaques formed by some appropriate aliquot of the phage preparation.



Bacteriophages do not exhibit a measurable independent metabolism. Yet they do possess means for the hereditary transmission of characteristics and are mutable in the genetic sense of the term. Their entry into a host bacterium seems to channel bacterial metabolic activity into the production of increased numbers of phage. The organic chemical structure of phages is predominantly protein and desoxypentose nucleic acid, the latter composing as high as 50 per cent of the total solids. Experimentally bacteriophages are easily studied utilizing the ordinary paraphernalia of the bacteriology laboratory. Their number may be assayed quickly and with a high degree of accuracy. They are readily isolated in large quantities in pure form from bacterial cultures which may be grown on synthetic media. For all of these reasons bacteriophages have become favored objects for the study of basic problems in virology. Additionally, phages have attracted the attention of the bacteriologist, biochemist, and geneticist interested in the nature of biological multiplication and in the mechanisms of inheritance at the molecular level of organization.

A bacterial species may serve as host for numerous different phages, for example about 100 different phage strains are known to be capable of infecting *Escherichia coli*. Yet the range of host species invaded by any one phage is extremely limited, being restricted to a single species, and more commonly to particular strains of the host species. While a given phage can infect only strains of an organism possessing a common antigen, not all strains of a bacterial species possessing a common antigen will be infected by the same phage type. In other words bacteriophage in their host range can distinguish between bacterial strains that are antigenically indistinguishable.

Phages undergo mutation with respect to the strains of bacteria they are able to invade, this kind of mutation being restricted to a change in the range of strains within the host species infected. The known exceptions to this behavior are the mutations of bacteriophages cross-infecting species of *Salmonella* and other *Enterobacteriaceae* and the reputed transformation of staphylococcal phages to types acting upon strains of *Corynebacterium diphtheriae*. The former case is probably not unexpected since the separation of species of gram negative intestinal bacteria is often based on few and trivial character differences. It is probably significant that the host range of the mutating *Salmonella* phages includes only bacterial strains and species possessing a common antigen. Contrarily the extent of the taxonomic gap bridged by an originally staphylococcal phage infecting diphtheria bacilli is unique and unexplained.

The mutation of phages is spontaneous and is only known to occur within the infected bacterium, presumably during the period of synthesis of new phage substance. In general when mutation of the phage occurs a



mixture of normal and mutated types of phage are liberated from the bacterium. Most curious is the failure to induce mutation in extracellular phage by the application of mutagenic agents such as the nitrogen mustards and radiant energy. With intracellular phage only ultraviolet light has been reported to induce mutations, mutagenic chemical agents and high energy radiation proving ineffective. Yet mutagenic agents are not bland since they do inactivate both intra- and extracellular phage, that is, render phage non-infective or incapable of proliferation within the susceptible host. No reasons for the failures to induce mutations are known. It is also an astonishing fact that spontaneous mutations have not been reported involving qualitative changes in the antigenic characteristics of bacteriophages. This certainly does not duplicate experience with non-bacterial viruses and raises a question as to how important the mechanism of what has been called a mutation phenomenon is for the evolution of new phage types.

The greatest volume of work has been done with a series of phage strains or types, known as T, which are able to infect and lyse *Escherichia coli* strain B. The basic data concerning these phages are recorded in Table 40. It is evident that a correlation exists between the serological grouping of these phages, their morphology and compatibility, and an inverse relation of plaque size to the dimensions of the phage particle. *Compatibility* refers to the capacity of different phages to simultaneously infect the same bacterium.

If a susceptible bacterium is exposed simultaneously to different but compatible phages, both phages will infect the organism and increase in number. The proportion of the types among the phages released on lysis will closely approximate the proportions of the phages used for infection. Where the phages differ in more than one character recombinants appear as well as the parent infecting types.

Non-compatible phages are adsorbed by a susceptible bacterium but only one type will be released on lysis of the bacterium. The *excluded* virus type which is adsorbed on the bacterium does not increase in number but simply disappears from the system. In spite of this finding the excluded phage seems not to be without effect on the production of the favored virus, since the yield of the latter is often observed to be lower than when it alone is adsorbed by a bacterium. This unexplained phenomenon has been called the *depressor effect*. Adsorbed, ultraviolet-irradiated, inactivated phage also is capable of exerting a depressor effect.

Upon exposure of a bacterium to incompatible phages, the phage that is excluded is determined by three factors, the nature of the two viruses and the order and interval of time between the adsorption of the two viruses by the bacterium. Thus T2 phage tends to exclude both T1 and T7

TABLE 40  
*The bacteriophages of the T system parasitising Escherichia coli strain B.*

SEROLOGICAL GROUP	TYPE	MORPHOLOGY (SIZE IN m $\mu$ )		CROSS-RESISTANCE GROUPING OF HOST	ADSORPTION RATE	LATENT PERIOD	BURST SIZE	SENSITIVE TO		COMPATIBLE GROUPS
		Tail	Head					Osmotic shock	Supersonic energy	
I	T1	120 x 10	Sphere 50	A	Medium	13	180	No	Less	}
	T5	70 x 15	Sphere 90							
III	T2	100 x 20	Ovoid 60 x 80	B	Fast	21	120	Yes	More	}
	T6	100 x 20	Ovoid 60 x 80	C	Fast	25.5	250	Yes	More	
	T4	100 x 20	Ovoid 60 x 80	D	Fast	23.5	300	Yes	More	
IV	T3	No tail	Spherical 45 m $\mu$	D	Medium	13	300	No	Less	}
	T7	Like T3 $\rightarrow$	Large	D	Medium	13	300	No	Less	

No mutation of phages involving a change in type has been reported. The mutations commonly described involve changes in host range and the morphology of plaques or rate of lysis of the host.

Mutations in host range are designated by the letter *h*, and as is customary in the literature of genetics a + sign is used to designate a wild type. Thus the wild type T2 with respect to its host range would be designated T2*h*+, and its mutant T2*h*-. If more than one host range mutant is under consideration they would be designated T2 *h a*, T2 *h b*, etc.

Mutants which cause rapid lysis and form large sharply defined plaques are labeled *r*. Other mutants tending to inhibit lysis have been labeled *w*.

while T1 and T7 in mixtures are equally likely to be favored. If an interval of several minutes intervenes between the times of addition of the two phages, invariably the phage added last will be excluded. If the two phage types are added simultaneously chance determines which type will be excluded from a particular bacterium, the last type adsorbed being excluded. The phenomenon described has been called *mutual* exclusion.

Bacteria mutate in their capacity to be infected by particular types of phages. A bacterium which mutates to a state of resistance to a phage is designated by placing a bar after the name of the bacterial species or strain and listing the types of phage to which it is resistant. Thus a mutant of *Escherichia coli* B resisting infection by T1 phages is designated B/1. It should be noted that a strain of bacteria may acquire by a one-step mutation resistance to several rather than a single phage type, for example B to B/1,5, and that this cross resistance is not related to the serological group of the phage (see cross-resistance column in Table 40).

In mutating to a state of resistance toward a phage to which it was originally sensitive, the bacterial organism loses the capacity to adsorb the virus. This change is shown by the inability to decrease the count of a phage suspension upon centrifuging out resistant bacteria added to the phage suspension. An exception is the reported existence of resistant staphylococci able to adsorb specific phage. That the resistance of mutants of *Escherichia coli* B does involve a loss in an adsorptive mechanism and not a loss of capacity to support the synthesis of new phage particles has been neatly demonstrated by the use of a phage hybrid labeled T2 (4). The mutant bacterial strain B/2 which resists T2 can be infected by phage T2 (4). However the phage progeny which result from this infection are genotypically T2. Here then is clear-cut evidence that the resistant bacterial mutant has not lost the capacity to support the synthesis of phage to which it is resistant.

The interaction of a bacterium with bacteriophage may be considered to occur in three major successive stages, adsorption of the phage to the organism, the increase in number of phage particles, and the release of phage during lysis of the bacterium. Each of these stages will now be considered in turn. Unless otherwise indicated the information reviewed has been obtained in studies with the series of T phages for *Escherichia coli* B.

On collision of a phage particle with a susceptible bacterium the phage is adsorbed. In the case of a phage such as T1, which possesses a morphologically differentiated head and tail structure, the phage is oriented tail-first on the bacterial surface. Electron microscope pictures show that the infecting phage particle is actually fixed and held at the surface and does not penetrate as a whole into the bacterium. Adsorption of phage takes place equally well on living and metabolically inactive or dead bacteria.



These and other bits of evidence are justification for speaking of adsorption of phage to bacteria in the usual physical-chemical meaning of the term adsorption. The initial attachment of the phage to the bacterium requires cofactors: univalent cations for the even numbered T phages, inorganic bivalent cations for others, and tryptophane in the cases of certain mutants of T4 and T6. Clear-cut evidence indicates the action of tryptophane to be on the virus rather than the bacterial surface.

For a short period after the adsorption of the phage, the attachment of the phage is reversible. During this initial stage if a suspension of infected bacteria is violently agitated in a Waring blender the phage is removed from the bacteria, or correlatively, violent shaking following immediately after mixing a suspension of phage and susceptible bacteria prevents the adsorption of the phage. These effects do not depend on any harmful effects of agitation since adsorption at the normal rate is initiated immediately on cessation of the shaking.

The rate of attachment of phage to bacteria simulates the kinetics of a bimolecular reaction. From the dimensions of the phage and bacterium the kinetic energy (Brownian movement) of the reactants can be calculated, and it has been shown that the rate of diffusion of purified phage is a function only of this energy. Phages with tails, therefore, do not exhibit any motility. The validity of these data is supported by the finding that the size of the phage particle predicted from a knowledge of the size of the bacterium and kinetic data of the adsorption of phage to the bacterium is verified by direct measurement of the size of phage in electron microscope pictures. These basic data also make it possible to estimate the number of collisions between phage particles and bacteria per unit time and to relate this measurement to the actual rate at which phage is fixed to the bacterial surface. Such calculations indicate that every collision is effective. Therefore any and all portions of the bacterial surface appear to be equally capable of adsorbing phage. Yet this conclusion appears to be anomalous in view of three facts which suggest that some collisions should be ineffective: a given bacterium appears able to take up only of the order of 100 to 200 phage particles which number would saturate only a small fraction of the available bacterial surface; phages with tails are fixed tail-first and not all collisions of these phages with the bacterial surface can be tail-first if Brownian movement (random movement) alone were the driving force of collision; a given organism can be infected by a large number of phage types each of which apparently requires for its adsorption different substrates in the bacterium. While much remains to be learned before these conflicting observations can be integrated into a unified whole, one thing does seem clear: a simple picture of the bacterial surface as a mosaic of specific receptor sites for phages is unacceptable.

The fact that only a portion of the bacterial surface actually can be saturated experimentally with a given phage may be due to the phenomenon of *lysis from without*. When a large enough number of phage particles (about 100) are adsorbed to a bacterium, lysis occurs without any evidence of invasion of the bacterium and without any increase in the number of phage particles. It appears that the reversible stage of adsorption is followed by a second irreversible stage in which the phage induces changes of an enzymatic nature which disrupt the surface structure of the bacterium. Given a sufficient number of adsorbed phage particles, the bacterial surface may change sufficiently so as to be no longer capable of taking up phage and eventually lyses. In this connection it may be significant that in a suspension of phage and bacteria after about 99 per cent of the phage is adsorbed the rate of adsorption declines, and free phages are found which are adsorbed only slowly to fresh bacteria. Sedimentation and electron microscope studies suggest that these phages have increased in size, presumably by the addition of shreds of surface material from damaged bacilli.

Following the stage of reversible adsorption the phage is irretrievably fixed, and a portion of the substance of the phage enters the bacterium. This stage has been called *invasion*. Employing phage tagged with tracer isotopes it has been shown that the desoxyribonucleic acid of the phage enters the bacterium. About 80 per cent of the phage protein remains adsorbed to the bacterial surface, and the remaining 20 per cent is unaccounted for. This unaccounted for portion of the phage protein might be involved in an enzymatic mechanism for transferring the phage desoxyribonucleic acid into the bacterium.

The period between invasion and lysis of the bacterium with liberation of phage is called the *latent period*. Under a fixed set of environmental and physiological conditions the extent of the latent period is characteristic for a strain of phage. With certain types of phage in synthetic media the latent period may be longer than it is in non-synthetic media, and the increase in phage number may be decreased.

Invasion is followed by the inability of the bacterium to synthesize adaptive enzymes and to grow and to multiply. Bacterial chromatinic bodies are dispersed or otherwise modified depending on the nature of the infecting phage, and staining with basic dyes may become less intense. Nonetheless, the bacterium remains metabolically active as indicated by the lack of any changes in oxygen consumption. While the invaded bacterium continues to synthesize protein from the moment of adsorption of phage, the production of nucleic acid ceases. After an interval of time of the order of ten minutes nucleic acid synthesis is reinitiated. Only desoxypentose-nucleic acid is synthesized and at a rate as much as four times greater than



is characteristic of uninfected bacteria. However, the amounts of desoxypentose nucleic acid synthesized are no greater than would be expected for the combined production of pentose and desoxypentose nucleic acids by uninfected bacteria. Unlike the situation for nucleic acid the amount of protein synthesized by the infected bacterium is greater than is required for incorporation into the phage being produced. In *Staphylococcus muscae* infected with phage the excess of protein is twice the amount needed for virus synthesis. It is not known whether all this protein is typically phage protein or a mixture of bacterial and phage proteins.

Phage does not appear to be synthesized from specific precursor materials. Evidence indicates that the protein and desoxypentosenucleic acid of the infecting phage are broken down extensively into low molecular weight nonspecific substances, and a portion of this material then finds its way into newly synthesized phage by way of ordinary processes of metabolic turnover. The bacterial substance contributed to the phage is also broken down extensively before being partly incorporated into phage material. In this regard an important observation is the lack of any antigenic relationship between the infecting phage and its host species. In Figure 76 an attempt is made to summarize schematically the present information on the origin of phage substance.

To this point the use of the term *multiplication* to describe the increase in number of phage particles following infection of a bacterium has been intentionally avoided. This has been done to emphasize the lack of knowledge as to the mechanism of the increase in number and to make it clear that this is one of the great unsolved problems of the bacterium-bacteriophage relationship.

Since the phage particles released from an infected bacterium are like the infecting particle and since any changes can be described as spontaneous mutations in the same genetic sense as the term mutation is used for other organisms, there must be a genetic mechanism in phage resembling in its fundamental character the hereditary apparatus of other organisms. This mechanism cannot arise *de novo*, and by some method of multiplication it functions as the direct link between phage generations. The existence of genetic recombination in phages is striking confirmation of this concept.

In principle the experimental study of recombination in phages is similar to that employed for bacteria. When individual bacteria are infected with two compatible but otherwise distinguishable mutants, phage recombinants are isolated. The two recombinant types occur in equal numbers as would be expected in theory. Since the absolute numbers of recombinants isolated are characteristic for each of the different pairs studied, work with numbers of such pairs has enabled the construction of a genetic-linkage map for the T2 phage.



The genetic data, however, do not permit any intimate glimpse of the actual process of increase in number of phage particles. From much work of a non-genetic nature it is clear that the phage material entering into the bacterium does not synthesize new structural materials which then assume the morphology of a complete phage particle and proceed to multiply.

The latent period may be divided into two phases of about equal length. In the first or *dark* phase the infecting particle disappears, or at least no evidence for its persistence has ever been found. If the bacterium is ruptured during this stage phage particles or structures are not found by any of

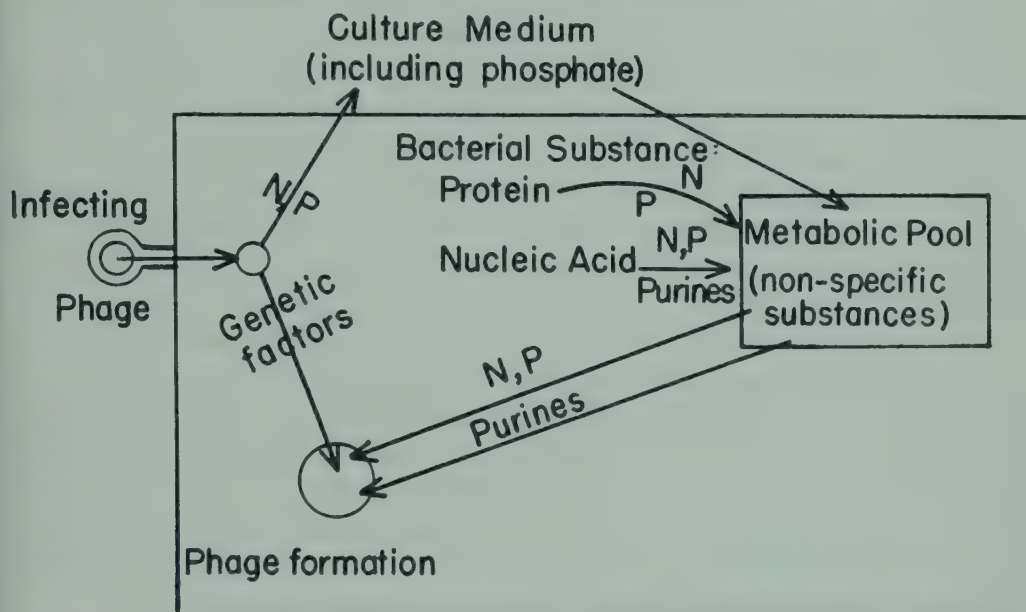


FIG. 75. A schematic representation of the sources of raw materials for the synthesis of new phage structure.

the methods used including direct electron microscopy. During the second half of the latent period or *maturation* period, particles resembling the phage appear within the bacterium. With T3 which is resistant to supersonic vibration, phage particles are not released by sonic vibration from the infected bacterium in the first two-thirds portion of the latent period but are in the final third of the period of time preceding lysis. From this and other evidence it would appear that not all the structures of the phage are synthesized at the same time. Possibly the maturation period represents a final stage during which preformed structures are brought together into the organized and infectious phage particle. In broad outline the process of phage formation would appear to proceed as follows. The omissions and ambiguities in the description result from lack of knowledge and indicate the questions which remain to be answered.

The portion of the infecting phage entering into the bacterium on invasion is broken down to its irreducible minimum of specific structure, that concerned with maintaining hereditary continuity. There has been some thought that this structure may break down into its parts, each of which might reproduce before being again brought together as a whole. Evidence for this view has not been substantiated. The structure concerned with heredity must multiply, if genetic data are to be taken at face value. Whether it multiplies as a master particle giving rise to non-multiplying progeny or multiplies to yield progeny each of which in turn are capable of multiplication is an unanswered basic question. Preceding or during this increase in the number of the basic structures which will serve as the hereditary apparatus of individual phage particles, other portions of bacteriophage structure are being accumulated *de novo* by processes poorly understood. Among other things it is not clear whether this synthesis is organized around some genetic locus which will become the individual phage or whether portions are made apart from one another and then integrated into a complete whole somewhat in the analogy of the building of a prefabricated house.

It is interesting that the duration of the latent period is independent of whether the bacterium is infected with one or more phage particles. If all the phage particles in multiple-infection were active in contributing to the increase in phage and did this by some process of uncomplicated multiplication, the rate of increase in number of phage within the bacterium should be by a geometric progression, and the latent period should be shortened as the number of infecting particles increases. It remains to be definitively proven that increase in phage is by a geometric progression.

The fact that the latent period is not shortened by multiple infection argues against a role for all of the infecting phage in the increase in phage number. Yet this conclusion is at variance with other kinds of information. Using isotopically tagged phage it has been shown that within the first five minutes of adsorption, multiple-infected bacteria treat all the adsorbed phage alike. Most important is the realization that the recombination phenomenon could not take place if only one phage particle per bacterium contributed to the increased number of phage. The idea that more than one phage particle can contribute to phage multiplication in the bacterium is also corroborated but is not of itself proved by certain irradiation experiments with X-rays. Single-hit curves are found to describe X-ray inactivation of the capacity to form phage by bacteria infected by a single phage while multiple-hit curves result with bacteria infected with more than one phage particle.

Following the maturation period the bacterium lyses and releases a characteristic average number of infectious phage particles. This number is



known as the *burst size*. It is emphasized that while the burst size may be a characteristic average number the yield per bacterium is variable. Thus with T1 the variation recorded is from 25 to 1000 particles per bacterium. The mechanism of lysis, probably enzymatic in nature, remains to be elucidated. The wall of the bacterial organism is actually dissolved away. Phage lysis is not a simple mechanical break in the bacterial cell wall followed by an osmotically induced extrusion of the intracellular contents.

A bacteriophage may be said to have several properties. It is specifically adsorbed by a host, it inhibits bacterial multiplication, increases in number, and causes bacterial lysis. What portions or structures of the phage particle are responsible for these different properties? In the case of the T2 phage it has been possible to experimentally study this question. This phage is *osmotically shocked*, that is, by rapid dilution of a phage suspension in two molar sodium sulfate solution or in concentrated glucose or glycerol solutions, the head and tail structure of a phage is preserved but the contents are extruded. The resulting *ghost* which can be cleansed of nucleic acid and seems to be composed exclusively of protein is specifically adsorbed by host strains of bacteria. Following this adsorption the bacterium is no longer capable of multiplication and it finally lyses. Here is conclusive evidence that the various properties of the phage can be attributed to specific phage structures. The nucleic acid of phage seems only to be concerned with the increase in number of phage particles. The phage ghosts are also capable of excluding noncompatible phages from a bacterium and compatible phages as well if they are applied sufficiently ahead of complete phage. This as well as other evidence indicates that both the non-compatibility of phage, and the exclusion phenomena generally are concerned with properties other than the capacity of the bacterium to support phage reproduction.

*Lysogenicity* is an interesting property of certain strains of bacteria. A lysogenic bacterial strain is one able to carry a phage to which it itself is resistant. Liberation of phage from lysogenic bacteria naturally or by ultra-violet irradiation, the addition of certain metabolites, reducing substances, and inorganic ions, yields typical phage for non-lysogenic but sensitive strains of the host species. The phage carried within lysogenic bacteria has been called *probacteriophage* to distinguish it from typical infecting phage. The release of phage from lysogenic bacteria by ultra-violet light has been likened to a trigger mechanism in which the irradiation acts to stimulate the development of probacteriophage to bacteriophage which is able to lyse the host organism.

Freeman (1951) has made a startling observation relating the virulence of the diphtheria bacillus to lysogenicity. If a culture of an avirulent strain is exposed to a phage to which it is susceptible, lysis ensues except for a



few organisms which grow out in the presence of the phage and prove to be both virulent and carriers of the phage, that is, lysogenic. Experimental evidence indicates that the observations cannot be explained by assuming the presence of small numbers of virulent mutants within the initial population of the avirulent culture exposed to the bacteriophage. It would appear as though a *directed* mutation is involved, the presence of phage guiding mutation from a state of avirulence to one of virulence. Since directed mutations have not been established in other systems, this case is of great importance if it is proved to be such a phenomenon.

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## CHAPTER XIII

# Bacterial Metabolism

It has already been pointed out that the nutrition of bacteria provides foodstuffs filling two needs: materials for the construction of protoplasm and materials serving as energy reservoirs for the endergonic physical and chemical processes of the organisms. In the present chapter an effort is made to discuss various aspects of these two general problems from a broad point of view rather than from a detailed one. This course is deemed advisable for several reasons. Many of the individual fields of knowledge are under such intensive investigation that the details are being expanded at rates beyond the ability of a textbook to keep up to date. Fortunately, there are a number of excellent reviews on these same subjects, so that little is to be gained by attempting another of the same type. Furthermore, the present authors have made an effort to limit details insofar as possible to cases illustrating principles.

Returning to the two roles played by foodstuffs, it may be anticipated that this classification is like many others in falling short of exactness. As a matter of fact the production of both energy and useful compounds is so intertwined in the metabolism of organisms that a neatly cataloged discussion cannot be presented. The widespread merging of the two different functions will be manifested in the following pages.

### ENERGY AND BACTERIA

Turning first to energy let us consider its production, utilization, and loss during bacterial activities. Energy sources and requirements under various conditions must necessarily also be discussed and related to chemical processes of fundamental importance.

Many bacteria are capable of physical movement, they all produce heat, some produce light, all carry out endothermic syntheses and need energy for multiplication and operations against osmotic gradients. Such processes require energy which often must be of a particular type. The latter condition introduces the additional requirement of special mechanisms capable of transferring the needed energy to the right loci in the right form and at the right time. A situation imposing such complex conditions as these implies a need for complex mechanisms, and this implication is fulfilled. Actually the intricacies are so great that we are only just beginning to visualize some of the simpler aspects of the problem.

## THE ECONOMICS OF BACTERIAL ACTIVITY

Since bacterial activities involve both matter and energy, very general statements may be made concerning each. One may conveniently describe the effect of bacterial organisms on their environments by means of the following balances.

$$\text{Foodstuffs} + \text{organisms} = \text{products} + \text{organisms}^* \quad (1)$$

where organisms\* represents the final individuals. These, of course, may differ from the initial organisms in number, size, shape, composition, or some combination of these factors.

Reaction (1) is really a material balance, meaning that every atom on the left side must appear on the right side, and no atoms not originally present may occur after the process has ceased. It is evident that the reaction does not involve a time factor, and does not consider rate. In general one cannot readily evaluate the full reaction which in any event gives no information on the mechanisms of the various processes submerged in the overall balance. Therefore, it is much more common to search for individual processes. When a balanced reaction is deduced for such a process, it is customary to seek out the sources of the reactants and to discover the subsequent products. Thus an increasing number of the component steps become known, and the general picture gradually emerges as a sum of the parts.

Studies have been made of the assimilation of carbohydrates into cellular components. With *Escherichia coli* and various simple sugars as sole sources of carbon, approximately 60 per cent of the carbon of the sugar consumed may appear as new cellular carbon. This surprisingly high efficiency of conversion is even more astonishing when one attempts to visualize the numerous steps that may be involved in the metabolic transformation of glucose. Furthermore, some of the assimilated carbon is certainly in molecules having a greater state of reduction than does carbohydrate. This extra reduction requires additional energy which can only be supplied by oxidation of a part of the carbohydrate. Such energy is not then permanently lost to the organism but rather is stored chemically in the reduced compound for future use.

An energy balance analogous to the above material balance may be written as:

$$E_{\text{foods}} + E_{\text{organisms}} + E_{\text{physical}} = E_{\text{products}} + E'_{\text{organisms}} + E'_{\text{physical}} \quad (2)$$

Here  $E_{\text{physical}}$  includes all forms of energy other than chemical that are supplied the system. The commoner additions would involve heat, mechanical energy, and light. Light has a profound effect on the energy balance



of the photosynthetic organisms since much of it is absorbed and stored in new structures as chemical energy. In other bacteria the light absorbed would be degraded to heat and appear in  $E'_{\text{physical}}$  as a product of the reaction. Only among the luminescent bacteria will light appear on the right hand side of the energy balance since light emission can only occur when a particular mechanism is available for converting chemical energy into visible radiation.

Heat or other energy must be added initially or no reaction can occur and the bacteria will not grow. Such needed energy may come from an incubator, from the kinetic energy initially possessed by the system, or from any outside source. This situation might be compared with the need for energy of activation discussed earlier in connection with the effect of temperature on bacteria. The heat liberated by bacterial activity will be considered in the succeeding section.

Frequently mechanical energy is applied to systems of bacteria, usually for purposes of agitation. Most of the energy thus applied appears subsequently as heat. Conceivably some of this heat might be converted into chemical energy and retained in this way, but actually by far the most of it is lost by the usual mechanisms of heat transfer, conduction, convection, and radiation. On the product side of equation (2) mechanical energy may appear in the motion of the organisms and in the normal course of events will be converted into heat and largely lost.

The molecules of the foodstuffs and the organisms themselves all contain a certain amount of potentially available chemical energy. The processes of life involve chemical reactions by which chemical energy is transferred from some molecules to others by withdrawing increments at various steps which supply energy as heat, movement, chemical energy for other steps, and sometimes as light or electricity. As a general result, life produces chemical reactions whose combined effects are a reduction in the potential chemical energy in the system.

One might logically anticipate that the organism capable of making the greatest quantity of energy available for its needs in a given environment would be in the best competitive position. This expectation appears to be met at least when organisms having similar capabilities are involved. When different species utilize different foodstuffs in any one environment, they do not compete except perhaps for space or through the effects produced by their toxic products of metabolism. Under such conditions the flow of energy through a particular portion of a system plays a much less important role than it does when different species are consuming the same raw materials.

As noted earlier, bacteria are unusual in their ability to oxidize foodstuffs and grow. They produce new cellular material at a remarkable rate and

appear to be limited only by the availability of foods and the accumulation of poisons. Actually these limitations apply equally well to all other organisms, some of which can produce protoplasm at high rates also. There is still another key limitation of multiplication rate that is well recognized among the organisms that reproduce sexually; namely, the number of ancestors. In this instance the term multiplication rate is used in the sense of increase in the number of individuals per unit time. A little reflection will show that this limitation must necessarily extend also to bacteria. However, it is customary to avoid this complication by thinking of multiplication rates in terms of units of population instead of merely as new individuals per time interval. Thus it seems clear that bacterial growth resembles other growth in the qualitative features of its economy although there may be quantitative differences.

Arguments formerly brought to bear on the question of the efficiency of bacteria may now be challenged. Whereas it was formerly proposed that bacteria were inefficient in their organization because they were capable of attacking many substrates not found naturally, the opposite stand may be taken. For example, some of the facultative anaerobes are able to utilize chlorate as a terminal oxidant. This powerful oxidizing agent is not a natural compound, and its use by some bacteria has been regarded as evidence that these species contain an enzyme that normally can never be used. Hence the charge of inefficiency has been brought. Moreover, the reduction product of chlorate is chlorite, an ion known to be bactericidal. The appearance of this product has been interpreted as being almost a deliberate attempt at suicide.

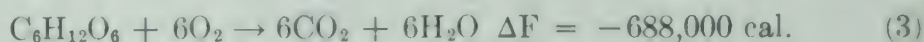
It is possible, however, to reinterpret some of these facts and to come to a contrary conclusion. In the first place it may be asked which ion is more dangerous, chlorate or chlorite? May not reduction of the chlorate represent an achievement in detoxication of an extremely dangerous material? Actually the question can be answered only indirectly and then only by observing the relative effects of the two materials on organisms incapable of detoxifying either of them. Unfortunately, since such studies are not reported it is difficult to come to any truly valid conclusion.

On the other hand, the presence of a system capable of utilizing unnatural substrates is no longer considered as either particularly unusual or wasteful, for it seems that enzymes may be formed adaptively to fit many different types of environments. If bacteria are capable of this activity more than other kinds of organisms, then they become able to grow in a wider variety of environments and would seem to be more efficient than less adaptable organisms. It may be, however, that bacteria are not especially unique in this regard since an increasing number of cases of activity on unnatural substrates are being found for higher forms of life. As an ex-



ample, the discovery of an enzyme in rabbit liver capable of decomposing nitromethane may be cited. Once again it would seem that the differences between bacteria and other organisms are probably quantitative rather than qualitative. In conclusion it does not appear that bacteria are very markedly more or less effective than are other forms, nor are they necessarily cluttered up with wasteful and unnecessary enzyme systems.

There is a growing body of opinion to the effect that living organisms are capable of surprisingly high efficiencies in utilizing the energy contained in substrates like carbohydrates. For example, the free energy<sup>1</sup> of oxidation of glucose is a little less than -690,000 cal. per mole when carried completely to carbon dioxide and water:



When an organism carries out reaction (3) the entire quantity of energy must be accounted for either in useful forms or as wasted energy. Some evidence has been adduced that for each molecule of oxygen consumed as many as six to eight high energy molecules are produced. The nature of these special molecules is discussed elsewhere (Phosphorus in Energy Transfer), but suffice it to say here that each such type of molecule yields approximately 10,000 to 15,000 cal. per mole when one chemical bond is broken by hydrolysis. The energy yield then amounts to about 450,000 cal. or 65 per cent of the total free energy which has been converted to at least 38 and perhaps 48 new high energy molecules that can be used in turn as energy sources in cellular processes. This figure of 65 per cent may be a maximum value since such high yields of high energy molecules have not always been obtained, but in any case the efficiency is actually rather high.

In the absence of oxygen the oxidation of glucose apparently proceeds only part way with a much diminished free energy change.



Thus anaerobic metabolism provides only a small fraction of the energy available during complete oxidation. It may be said, therefore, that organisms utilizing reaction (4) are inefficient. Be this as it may, only about 20,000 cal. are transferred to high energy molecules, and the remaining 60 per cent is evidently degraded to heat and lost. To this extent fermentation is clearly inefficient. However, since the heat produced normally brings

<sup>1</sup> Free energy ( $\Delta F$ ) is a thermodynamic term referring to the energy available from a given reaction or series of reactions when carried out at constant temperature. A negative sign for the free energy change accompanying a reaction indicates that the reaction will go and that energy will be liberated. A positive sign indicates that a given reaction will not occur unless energy is supplied.



the temperature of the medium above that of the environment the heat loss need not be considered as entirely ineffective since many of the metabolic reactions will be accelerated at the elevated temperature.

#### HEAT PRODUCTION BY BACTERIA

As do other organisms, living bacteria produce heat, leading under some conditions to a marked rise in the temperature of the culture. Ordinarily a maximum rise is observed only when conditions are favorable for rapid growth and when the culture is so insulated as to greatly retard heat transfer. In nature such conditions occur during the storage of moist organic matter, e.g., green hay, manure piles, damp grain, accumulations of dead leaves.

The heat evolved must have its source in metabolic activities and thus be traceable directly to the substrates attacked by the organisms. However, the details of the processes involved are speculative in nature, and there is but little knowledge of the fraction of the free energy of exergonic metabolism ultimately degraded to heat. Although much calorimetry has been applied to studies of the total energy (or heat content) of various human foods and to the utilization of such energy by animals, little work has been done with bacteria.

Heat does appear in pure cultures of typical bacteria and produces a temperature rise. Whenever the system is designed to permit a great total growth and a correspondingly great evolution of heat into a culture insulated to prevent heat loss, a maximum temperature is reached beyond which the system apparently cannot rise. Tentatively this limitation has been correlated with the heat lability of enzymes. In fact the maximum temperature due to growth in insulated systems has been found to vary with different species and strains, and these variations have been found to correlate with variations in the sensitivity of some of the enzymes present. It has been proposed, therefore, that growth occurs under favorable circumstances and raises the temperature until certain essential enzymes are adversely affected and that no further temperature rise is then possible. This concept does seem logical since one would anticipate that a temperature should be reached at which enzymes are partially inactivated and metabolism is correspondingly diminished. In other words a steady state appears that depends upon heat production, heat loss, and the temperature effects on catalytic activity.

As far as the individual bacterium is concerned even less is known concerning heat production, for populations must be examined since the heat liberated by one organism cannot be measured. Cultures have been studied calorimetrically in correlating heat production with the growth curve. With *Escherichia coli* for instance heat is produced throughout the life of

the culture at least into the stationary phase. Although fission ceases, metabolism continues as long as the bacterium lives, and heat is produced at a low rate per organism.

During rapid multiplication the heat produced per organism is much greater than during either the lag or declining growth phases. From such observations it appears that heat and metabolism are very closely allied, rising and falling together. It might also be expected that heat production and multiplication are parallel, but this relationship is apparently not strictly obeyed as may be seen in Figure 77. Heat production rises more rapidly than cell production during the early portion of the exponential phase of growth. During the last part of the exponential phase, it falls sharply. This apparently means that the bacteria are more active at evolving heat in the beginning of cultural development than at making new organisms and that this trend is later reversed. When the rate of division begins to diminish, the production of heat parallels multiplication for some time and then rises sharply as the formation of new individuals becomes quite low. Presumably the curve will rise to infinity when multiplication ceases completely.

These observations indicate that heat production and bacterial multiplication cannot be completely related. Unfortunately there are no experiments which make it possible to decide whether there is any real connection between growth and the reactions leading to the production of heat. However, if one correlates oxygen consumption (see fig. 33, Chapter 8, page 248) with heat production shown in Figure 77 of the present chapter (note one quantity is arithmetic and the other logarithmic), metabolism and heat show a similar behavior throughout the culture growth cycle. Clearly data are needed to develop the point. With the techniques of both manometry and calorimetry now well developed the design of the experiments should be similar in principle to the method used in the investigations cited below.

When a substrate is metabolized in the absence of available nitrogen so that multiplication is prevented, part of the substrate is oxidized and energy obtained. A portion of this energy is utilized chemically by the organism and part is converted to heat and dissipated. Yeast with glucose as a substrate produces heat corresponding to 25 per cent of that expected from the complete oxidation of the same amount of glucose dissimilated. The energy not appearing as heat either is used in driving reactions forward or never actually appears because part of the glucose or some of its partial degradation products are incorporated into the cell. As will be seen later, organisms may gain in weight though not in number when nitrogen is withheld and an oxidizable substrate is present in their environment. This indicates that part of the dissimilated substrate is converted into cellular

material; in other words, not all is utilized for the immediate production of energy. Although heat studies of this type have not been performed with

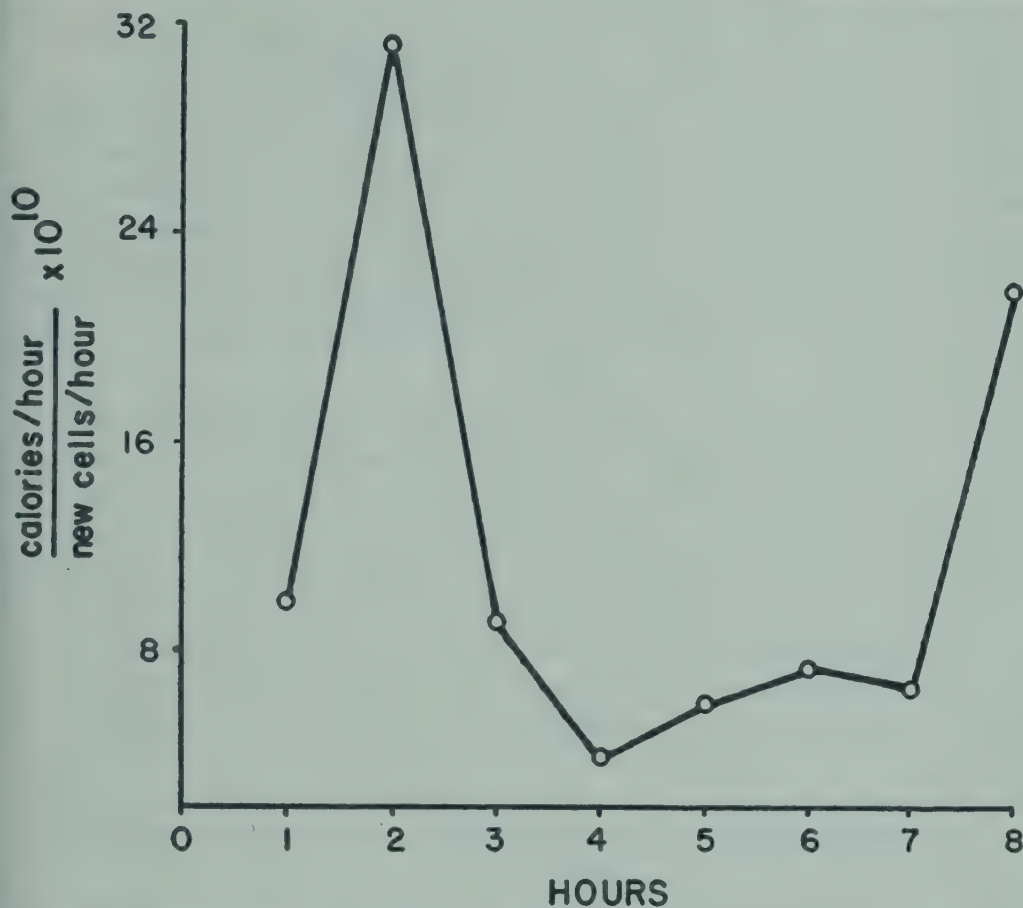


FIG. 77. Dissimilarity in the rates of multiplication and heat production by *Escherichia coli*. This method of plotting is chosen to emphasize the differences.

The data at the later times are somewhat uncertain because errors in cell counts become more important as the number of new individuals becomes smaller. However, the rise at the end must be qualitatively real because data on the heat evolved was taken at nine and ten hours and showed an essentially constant production of heat in this zone. Unfortunately, bacterial counts were not made at these times but presumably multiplication had nearly ceased. These considerations indicate a high rate of heat production per new bacterium in the stationary phase.

(Calculated from data of Bayne-Jones and Rhees, 1929)

bacteria, the general findings would probably be similar to those obtained with yeast.

#### PHOSPHORUS IN ENERGY TRANSFER

A major problem of life is the transfer of energy from a source to products and the utilization of part of the energy for growth, multiplication,



and other biological processes. Organisms are amazingly successful in this activity, and they show relatively little variety in the general procedures employed.

One of the principal goals of comparative biochemistry has been the discovery of the common processes of life possessed by different forms. In at least one direction the goal has been reached, namely, in the elucidation of the general mechanism of energy transfer. For this purpose phosphorus compounds appear to play a universal role and are involved in energy transfers in all the different species that have been examined. While the role of phosphorus in energy transfer seems to be one of the important common denominators of life, that does not mean that energy is not transferred without it. A brief discussion of other processes appears in the following section.

Studies of the energy requirements of great numbers of chemical reactions have taught that a net amount of energy is either consumed or liberated in nearly all. Indeed, those reactions which apparently do not require or evolve energy under one set of conditions do so under other conditions. Some reactions involve quite small energy changes, others extremely large changes, and still others lie everywhere in between. The reactions of biological systems might be expected to lie over the entire energy range. It seems, however, that individual reactions in biological processes involving enormous energy changes probably do not occur. Rather the overall processes of living things like the complete oxidation of glucose appear to take place in a number of steps. Each of these steps involves free energy changes but in small increments whose sum equals the effect of an overall one-step process.

Many of the individual steps which involve comparatively large increments of energy and which evolutionary processes have not altered by subdivision into several steps depend upon the participation of compounds containing phosphorus. While there are many other known systems not involving phosphorus and with the desired energy content, these do not seem to be utilized biologically. In seeking an explanation of this limitation one finds it outside the province of thermodynamics, a discipline which concerns itself chiefly with the properties of systems at equilibrium or with the total changes occurring when a system goes to equilibrium. Thermodynamics tells us that many different substances may be suitable for a given reaction. It does not, however, predict whether the reaction rates will be suitable, and for this latter information the aid of *kinetics* must be invoked. This discipline deals with rate processes like motion or chemical reaction.

Although the kinetic information is less complete in some respects than that of thermodynamics, it is now felt that the universal role of the phos-

phorus compounds stems from unusually desirable thermodynamic and kinetic properties. Table 41 serves to classify typical high energy compounds whose thermodynamic instability in water is similar. The reactions concerned may be represented in the general form.



While the free energy changes of the actual reactions are somewhat uncertain and may differ, they probably lie between  $-10,000$  and  $-15,000$

TABLE 41

*A comparison of the kinetic instability of typical molecules that are thermodynamically unstable with respect to hydrolysis*

NAME	FORMULA	HALF-LIFE IN WATER
Acetic anhydride	$\begin{array}{c} \text{O} \quad \quad \text{O} \\ \parallel \quad \parallel \\ \text{CH}_3-\text{C}-\text{O}-\text{C}-\text{CH}_3 \end{array}$	A few seconds
Acetylphosphate	$\begin{array}{c} \text{O} \quad \quad \text{O}^- \\ \parallel \quad \diagup \\ \text{CH}_3-\text{C}-\text{O}-\text{P}=\text{O} \\ \quad \quad \diagdown \\ \quad \quad \text{O}^- \end{array}$	Several hours
Pyrophosphate	$\begin{array}{c} \text{O}^- \quad \quad \text{O}^- \\ \diagdown \quad \diagup \\ \text{O}=\text{P}-\text{O}-\text{P}=\text{O} \\ \diagup \quad \diagdown \\ \text{O}^- \quad \quad \text{OH} \end{array}$	Very long

calories. This magnitude places them as all quite unstable thermodynamically in spite of the differences in kinetic stability.

Acetic anhydride is typical of the high energy substances which react so rapidly with water that little of it could possibly take part in metabolically more useful reactions. Acetylphosphate reacts much more slowly with water and could participate in many more biological reactions in the presence of water than does acetic anhydride. Finally, pyrophosphate is indefinitely stable in cold water although its hydrolysis is potentially probable and would be far in the direction of complete hydrolysis. This material should be ideal in any reactions that proceed spontaneously at useful rates or that can be so catalyzed as to increase the rate of a desirable reaction.

Some of the rather extensive list of known phosphorus compounds of metabolic importance are listed in Table 42. These free energy data are not all extremely accurate, but the general range is about correct in all

cases. Where equilibrium measurements have been made the difference between two materials is accurately known. In the table the result of such measurements are indicated as differences with respect to a standard reference material (adenosinetriphosphate). It will be seen that the first four compounds listed are either typical esters or glycosides. These all are relatively stable toward hydrolysis. It has in fact been suggested that the hydrolysis of such low energy substances results in very little free energy

TABLE 42

*Free energy changes on hydrolysis of certain metabolically significant phosphorus compounds*

COMPOUND	$\Delta F^\circ$ OF HYDROLYSIS	pH	°c
$\alpha$ -glycerophosphate.....	-2200 cal	8.5	38
Glucose-6-phosphate.....	-3000	8.5	38
Glucose-1-phosphate.....	-4900		
Adenylic acid .....	-2000 to -3000		
ADP.....	= ATP		
ATP.....	-10,500	7.8	20
DPN <sup>+</sup> .....	= ATP (?)		
Phosphopyruvate.....	= ATP - 4,300	8.4	20
Acetylphosphate.....	-15,000 (?)		37
1,3-diphosphoglyceric acid .....	= ATP - 4,600	6.9	25
Creatine phosphate.....	= ATP - 1,100	7.7	20

ADP = adenosinediphosphate

ATP = adenosinetriphosphate

DPN<sup>+</sup> = diphosphopyridine nucleotide

$\Delta F^\circ$  values related to ATP indicate that the free energy change is that of the hydrolysis of ATP with the indicated numerical change. For example, phosphopyruvate is less stable thermodynamically than is ATP.  $\Delta F^\circ$  data refer to free energy changes in the standard states in which the quantities involved are one mole of each material.

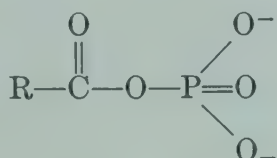
change when all the compounds involved, including water, are in standard states at concentrations of one molar. The observed values of a few thousand calories are attributed to the high concentration of water which tends to drive the hydrolysis forward.

The last seven compounds in Table 42 are called high energy substances because their hydrolyses result in large free energy changes. Five of them are acid anhydrides; that is, they are hydrolyzed with the formation of two new acidic groups and as is the case with other anhydrides a good deal of energy is liberated in the process.

Why should such large quantities of energy be involved in the reaction of these latter compounds with water? There appear to be several reasons.

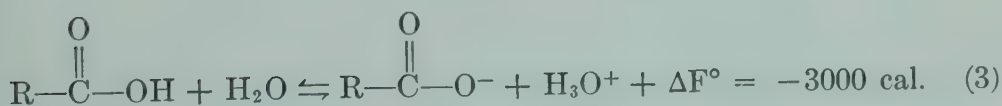
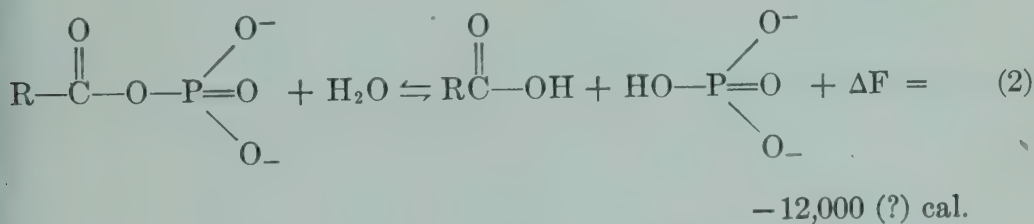


tributing factors with more than one operating in any given case. Of these, resonance apparently participates in most cases. When resonance exists in a structure, that structure is stabilized and shows a reduced reactivity. This phenomenon then reflects abnormalities in the properties of molecules making them somewhat different from those expected for the structural formulas that are usually written. It is possible, however, to bring together into one molecule two structures whose characteristic resonance properties are incompatible. The resulting interference is sometimes called *opposing resonance* and reduces the stability of the complete molecule toward reactions tending to separate the groups having the interfering effects. For example, in the acylphosphates



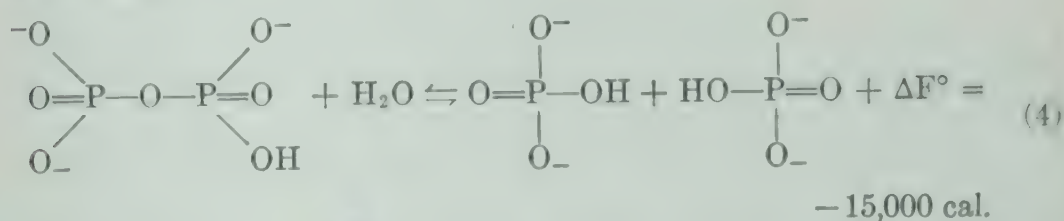
the important reactive structures are the carboxyl and the phosphate groups. The resonance of each interferes with that of the other when they are brought close together as in the acylphosphates making this product unstable toward hydrolysis. Opposing resonance also appears to be an important factor in the instability of the pyrophosphate bonds of ADP, ATP, DPN<sup>+</sup>, and of pyrophosphate itself.

In many of the high energy compounds additional free energy change derives from the ionization of the groups formed by the reaction. The carboxyl group appearing when acylphosphate is hydrolyzed ionizes and drives the reaction further toward the right.

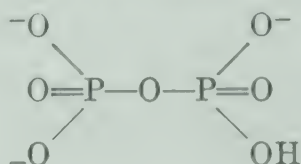


For the overall reaction, the sum of (2) and (3), the contribution of the ionization step is quite appreciable at pH 7. A similar reasoning may be

applied to the hydrolysis of pyrophosphates in which an additional ionized group appears.

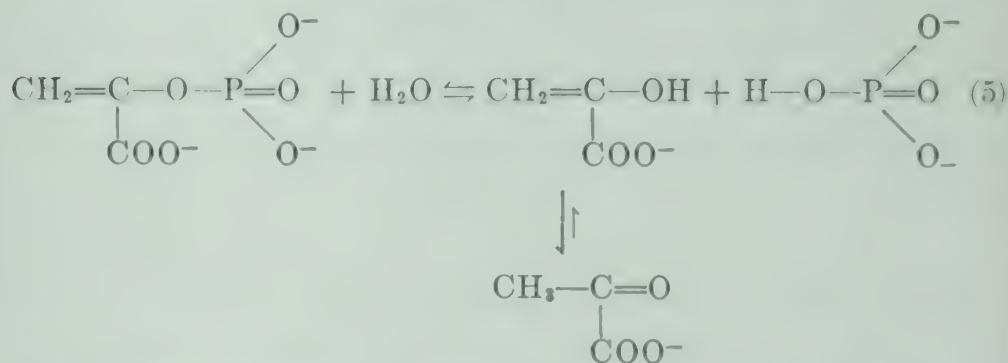


A third factor contributing to the instability of some of the high energy phosphates may be illustrated by pyrophosphate. In this molecule



three of the hydroxyl groups are ionized at pH 7-8, and the three oxygens thus exposed carry negative charges. It is now believed that the electrostatic repulsions existing between these nearby charged atoms decrease the stability of the molecule. It is as if the repulsions tend to tear the molecule apart. This type of instability makes an important contribution in ADP, DPN<sup>+</sup>, and the like.

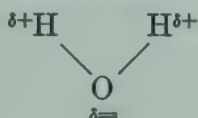
One other factor seems to affect the stability at least in the case of phosphopyruvate. In the hydrolysis of this compound a succeeding reaction removes most of one of the products and consequently drives the first step far toward completion.



Under ordinary conditions pyruvate is much more stable in the keto than in the enol form. The rearrangement step appears to have a  $\Delta F^\circ$  of -5500 to -9000 cal. Thus this second reaction with ketopyruvate as a product is quite important in the low stability of phosphopyruvate, one of the few high energy intermediate substances known in organisms that is not an

acid anhydride inasmuch as it does not yield new acidic groups on hydrolysis.

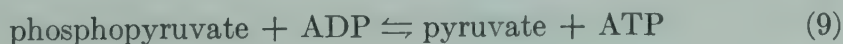
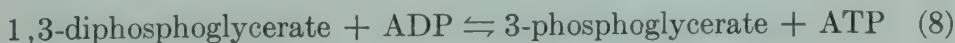
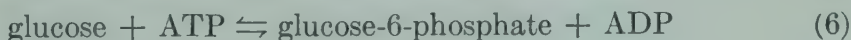
Although the above theoretical bases for the thermodynamic instability have been proposed, well substantiated reasons for the differences in the kinetic stabilities have not yet been advanced. In Table 41 the three compounds listed have similar values of  $\Delta F^\circ$  for their hydrolysis. They, of course, react at much different rates with water. Inspection of the formulas reveals a correlation between the kinetic stability and the number of negative charges grouped about the covalent bond that is susceptible to hydrolysis. Perhaps this accumulation of negative charges is responsible for the low reaction rates. If so, it must involve repulsion of water molecules probably due to the presence of partial negative charges on the oxygen atoms of the water. In this connection it should be recalled that there is a partial separation of charges in the water molecule making it both physically and electrically unsymmetrical as shown.



The charges involved here are smaller than typical ionic charges and are represented by placing the Greek letter  $\delta$  and the sign of the charge near the appropriate atom. While not yet proved, this hypothesis of a repulsion of charges seems to be the best so far presented.

Up to this point the discussion has been concerned not so much with the biological reactions in which these high energy phosphorus compounds participate but rather with their nature and general properties. Turning to the actual systems of biological importance one finds that the number of known reactions and general processes is quite large. No attempt will be made to catalogue, let alone discuss, all of these, but some typical examples of the various kinds of reactions will be presented.

Among the reactions which have been most extensively investigated are those involving ADP and ATP. Some typical examples are:

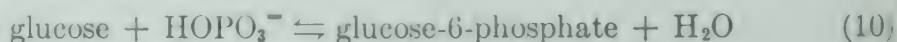


The various enzymes involved in these reactions all appear to require magnesium ions and free sulfhydryl groups ( $-\text{SH}$ ). Listed in the same order



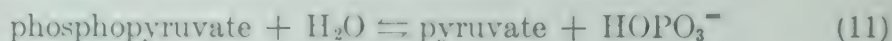
as the above reactions for which they are catalytically active, the enzymes are called hexokinase, phosphohexokinase, phosphoglyceric phosphokinase, and pyruvic phosphokinase.

Reactions (6-9) are steps in the dissimilation of glucose taking place in certain organisms when the carbohydrate is utilized as either an energy or a material source. In the first two processes energy in ATP is used to drive a reaction that will not go to any great extent without a coupled source of energy. Furthermore, the phosphate incorporated into the final products also comes from the ATP. As a result new compounds are formed that are required in the general metabolic processes but which have higher free energies than did the original reactant.



The  $\Delta F^\circ = +3000$  cal. for this reaction at  $38^\circ\text{C}$ . and pH 8.5 indicates that appreciable quantities of glucose-6-phosphate will not be formed unless energy is supplied. The ATP thus supplies both the driving force for the phosphorylation and the phosphate consumed.

Plainly the reactions like (6) and (7) which need ATP depend upon a continuous supply of this high energy intermediate. Reactions (8) and (9) are subsequent metabolic steps and illustrate how ATP is formed. In both cases the substrates are high energy phosphates capable of surrendering the energy via various reactions. For instance phosphopyruvate will react spontaneously with water yielding much energy:



$\Delta F^\circ = -15,000$  cal. at  $20^\circ\text{C}$ . and pH 8.4. Instead of allowing this energy to be lost as heat, it is trapped by the following reaction:



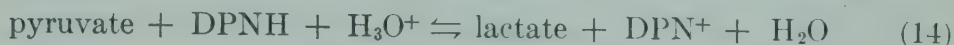
for which  $\Delta F^\circ = +10,500$  cal. If reactions (11) and (12) are combined the result is reaction (9) which is driven by a free energy decrease of about 4500 cal. Moreover, this reaction is drawn to the right by withdrawal of ATP in energy consuming steps like (6).

The complete oxidation of a glucose molecule to carbon dioxide and water yields a total of at least 38 new high energy molecules. Many of these are put to use by the living organism in the construction of cellular components that also have high free energies. Still others, also a sizeable group, are employed for various ultimately non-synthetic activities like light emission, mechanical energy for movement, and osmotic control.

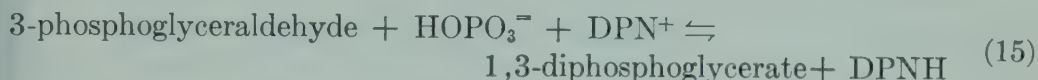
In addition to these reactions which transfer both energy and phosphate there are others which involve high energy compounds containing phosphate but in which the phosphate is not transferred. Typical of these are

some of the reactions of  $\text{DPN}^+$  (diphosphopyridine nucleotide) and flavin-adeninedinucleotide. These compounds are involved in especially important oxidations. All show high free energy changes on hydrolysis and are therefore called high energy compounds, but their participations in oxidation-reduction reactions are more important biologically than are the transfers of the phosphate contained in these molecules.

Typical bacteria appear able to convert malate into lactate and carbon dioxide for which process  $\text{DPN}^+$  seems to be necessary. It has been suggested that the mechanism involves a coupled oxidation-reduction mediated by  $\text{DPN}^+$  and represented by:



The reduction of pyruvate by reduced  $\text{DPN}^+$  (DPNH) goes far toward the right and pyruvate does not accumulate in the presence of the reduced dinucleotide. In other cases the fate of the DPNH is much different.



and the reduced dinucleotide may react with pyruvate as in (14) or perhaps be reoxidized by flavinadeninedinucleotide (FAD):



The  $\text{FADH}_2$  may then be oxidized by the cytochrome system (which will be discussed later) or by molecular oxygen:



Reaction (17) must be important in those bacteria which lack cytochrome and where hydrogen peroxide is known to appear. All these various oxidation-reductions illustrated require enzymes, and all involve energy changes leading eventually to final low energy products or to high energy components needed by the cell. The efficiencies seem to be at least 60–70 per cent with a part of the unutilized energy degraded to heat.

Reaction (16) is apparently much more complex than is shown. In it inorganic phosphate disappears and high energy phosphate is formed at the rate of one molecule per molecule of FAD reduced by DPNH. The mechanism of the process has not been elaborated although rather promising theories have been advanced. When  $\text{FADH}_2$  is oxidized through the cytochrome system by way of oxygen still more high energy phosphate is formed. Nothing is known of this phase except that it has been felt that the active phosphate does not seem to be formed in the cytochrome system. Therefore, one must conclude that the energy is transferred to phosphates



somehow during the oxidation of the  $\text{FADH}_2$  by the initial member of the cytochrome system.

The overall transfer of hydrogen from one DPNH molecule to oxygen yields three high energy phosphates and thus contributes in a major way to the general fund of energy transfers. Actually six molecules of DPNH and two of TPNH (which behaves in the same way) are oxidized for each molecule of glucose converted to carbon dioxide and water. Therefore, 24 high energy phosphate bonds are formed in processes starting from the oxidation of these reduced coenzymes and constitute a majority of the total of 40 known, emphasizing the importance of  $\text{DPN}^+$  and  $\text{TPN}^+$  in energy transfers. The known steps in which these two coenzymes are reduced have been incorporated into the general scheme of aerobic metabolism as set forth in the section on intermediate metabolism.

The high energy compound diphosphothiamine is involved in several metabolic reactions of interest, e.g., the oxidative decarboxylation of pyruvate to acetate and several decarboxylations. At present, however, this substance is merely known to be required, but the mechanism of its action is not understood. Uridine diphosphoglucose is presumably a high energy compound since it contains pyrophosphate. This material serves as a coenzyme in the reactions that interconvert various hexoses. The mechanisms of all of these processes are currently under intensive investigation.

Other kinds of phosphorus compounds are known to play metabolic roles in transferring energy, but these are so-called low energy materials, that is, they show a free energy change of only a few thousand calories when hydrolyzed. The known examples are required for one or more reactions although the details are not always understood. Riboflavinphosphate, for example, is clearly an intermediate in energy transfers and in bacteria participates in the oxidation of succinate. Others may or may not be primarily concerned with energy transfers. Finally the oxidation of succinate to fumarate yields two new high energy phosphates, but the mechanism is unknown except for the possibility of mediation by riboflavinphosphate which presumably acts in the removal of hydrogen from succinate and its transfer eventually to oxygen.

The origin of high energy compounds has been discussed and some specific examples of reactions in which these substances participate have been presented. It seems desirable, therefore, to complete the picture by attempting to tie high energy phosphate to the synthesis of cellular components. As a general rule these synthetic reactions yield products having higher energies than did the reactants involved and some sort of energy coupling would seem necessary for this to occur. The rather large number of biological syntheses now known to depend upon high energy phosphates



is presented in general form in Table 43. Specific examples are also included. Although all of these reactions are written as equilibria, in a few cases this may not be actually true. At any rate reversibility has been invariably found or else a separate mechanism that reforms the original reactants has been proposed to occur.

In most of the processes summarized in the table water is one of the products. Since the concentration of water in the actual systems is always high, energy is needed to overcome this concentration effect in order to accomplish extensive synthesis. The quantity of free energy required for this purpose is of the order of 2500 cal. which accounts for the endergonic nature of several of the reactions and contributes to all in which water is involved. The highly endergonic reactions (requiring considerably more than 2500 cal.) naturally are less affected by the presence of water than by other energy requirements.

Not much is known of the energetics of transmethylation, so the free energy requirements have not been estimated for these reactions. The fatty acid synthesis, seemingly spontaneous, requires high energy phosphate in a first step for which  $\Delta F^\circ = +16,000$  cal. A subsequent reaction is responsible for the overall free energy decrease but apparently cannot drive the first highly endergonic step.

No attempt is made herein to represent the exact mechanism of the coupling of high energy phosphate to synthetic reactions. In those particular syntheses for which details are known more complete mechanisms are presented later.

#### OTHER SYSTEMS THAT TRANSFER ENERGY

At the outset of this discussion it should be recalled that all reactions undoubtedly involve energy transfers of some sort from one molecule to another or from one part of the system to another. Furthermore, the quantities of energy involved may be large even when only small quantities are observed since the observer may be aware only of the net result. A net result may actually be the difference between two large numbers which represent energy transfers forward and backward during the reaction.

This idea may be illustrated using the concept of the energy of activation. It will be recalled from Chapter 10 that many reactions which will not occur spontaneously will proceed once initiated, and the energy of activation required to activate a few of the reacting molecules is recovered when the products appear. If the energy of the reaction is trapped, the process stops. Normally however, this does not occur and the energy of reaction is transferred to other reactant molecules having low levels of energy and which are thus activated in turn and react repeating the

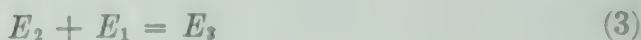
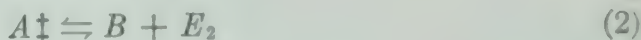


Keto acids	$\text{RCOO}^- + \text{R}'\text{COO}^- + \text{H}_3\text{O}^+ \rightleftharpoons \text{RCOR}'\text{COO}^- + 2\text{H}_2\text{O}$	+16000 to +17000	Pyruvate	<i>Escherichia coli</i> , <i>Staphylococcus aureus</i> , <i>Clostridium butylicum</i> , <i>Clostridium kluyveri</i> <i>Escherichia coli</i>
$\beta$ -Carboxylation	$\text{CH}_3\text{COCOO}^- + \text{HCO}_3^- \rightleftharpoons \text{}^-\text{OOCCH}_2\text{COCOO}^- + \text{H}_2\text{O}$	+5280	Oxaloacetate	<i>Escherichia coli</i> , pigeon, <i>Propionibacterium</i>
Fatty acids	$2\text{CH}_3\text{COO}^- + 4\text{H} \rightleftharpoons \text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O}$	+10,000	Butyric acid	<i>Clostridium kluyveri</i>
Citric acid	$\begin{array}{c} \text{CH}_3\text{COO}^- + \text{}^-\text{OOC}\text{COCH}_2\text{COO}^- \rightleftharpoons \text{HOCCOOH} \\ \begin{array}{cc} \text{CH}_2\text{COO}^- &   & \text{CH}_2\text{COO}^- \\ & \text{HOCCOOH} & \end{array} \end{array}$	+4680	Citrate	<i>Escherichia coli</i> , <i>Pseudomonas fluorescens</i>
Transmethylation	$\text{RSCH}_3 + \text{R}'\text{NH}_3^+ \rightleftharpoons \text{RSH} + \text{R}'\text{NH}_2 + \text{CH}_3$	—	Creatine Methylation of nicotinamide	Rat Rat
Aldehydes	$\text{RCOO}^- + \text{H}_3\text{O}^+ + 2\text{H} \rightleftharpoons \text{RCHO} + 2\text{H}_2\text{O}$	+16,000	Phosphoglyceraldehyde from the acid	<i>Clostridium butylicum</i>

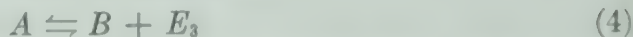
(Modified from Kaplan, 1951.)



cycle. The process may be outlined in a general way as follows:<sup>2</sup>



and the overall reaction is the sum of these three.



It seems that the values of  $E_1$  and  $E_2$  may be independently either large or small. Therefore,  $E_3$  may have either a large or small absolute magnitude meaning that the net quantity of energy involved may be great or little. Furthermore,  $E_3$  may be either positive or negative, i.e.,  $E_2 > E_1$  or  $E_2 < E_1$ . In one case a net amount of energy is liberated; in the other it is consumed. Sometimes  $E_2 = E_1$ , but in any case for every existing chemical reaction at least part of  $E_2$  is transferred to unreacted  $A$  to keep the process going. While the exact steps involved in these energy transfers are not known, the basic importance of these broad general ideas cannot be over-emphasized for all chemical processes including those of metabolism. In some cases mediators are known to play important roles. Of these the phosphorus compounds have been discussed in the foregoing section. Other important examples are included here.

Of the many reactions participating in such a complicated series of processes as metabolism a large fraction involve rather low net energies, and in these cases unique mechanisms are not required. At the low levels, the energy liberated during reaction may appear as heat while at high energy levels conversion to heat might be undesirable because of the possibility of local damage due to zones of excessively elevated temperature. Even more important, the endothermic reactions requiring a high energy could not occur because heat energy in the temperature range of life cannot be sufficient for all of the necessary reactions.

While the conversion to heat of the energy from low energy reactions may occur rather extensively, it is not so serious economically as would be a similar conversion in high energy reactions. Furthermore, since many other processes require energy in equally small amounts much of the heat produced by exothermic reactions may be utilized in driving the slightly endothermic processes. Actually such low energy coupling arrangements may not involve indirect transfers of heat, but the energy may be transferred more directly from one reaction to another occurring close by.

<sup>2</sup> In these expressions  $A$ ,  $A^\ddagger$ ,  $B$ ,  $E_1$ ,  $E_2$ , and  $E_3$  are respectively the reactant, activated (high energy) reactant, product, energy of activation, energy of reaction of the activated reactant, energy of reaction (overall process as for reaction (4)).

An increase in the rotational or vibrational energy of molecules could very well serve this purpose, and the energy thus held could be transferred to a reactant molecule by collision. In the usual instance of consecutive reactions the product of the first may contain the energy of reaction as activated molecules which are capable of undergoing a second and endothermic step without the addition of energy. These ideas may be illustrated as follows:



the energy  $E$  may be transferred indirectly as heat to the next step



by molecular collisions. However, if reaction (5) directly precedes another, energy need never be exchanged:



where  $B^E$  represents the activated product of the first step. Processes of this type are likely to be quite important in biology where reactions follow each other in cycles or catenary series.

Chlorophyll and the pigments performing related photosynthetic functions are key materials in energy transfers. They are able to absorb light and to pass the energy thus obtained along to water without the known intervention of phosphorus compounds. The mechanism of this important type of energy transfer is only partly understood although the object of intensive research. Apparently some of the biological systems participating do so across phase boundaries since both solid and solution phases are involved in photosynthesis. In the non-photosynthetic organisms light cannot be used as an energy source, so these forms must rely completely upon dark reactions for their energy. All such cases require oxidations during which energy is transferred by mechanisms that have been already discussed in general terms.

All biological systems carry out *terminal oxidations*, by which one refers to the last oxidation-reduction step which the given system is able to accomplish. Anaerobic dissimilation frequently stops at an acid or an alcohol beyond which point the oxidation cannot be carried, and the terminal oxidation thus does not involve oxygen. *Clostridium thermoaceticum*, for example, ferments glucose to acetic acid as the only product:



Needed energy is obtained by oxidation of one part of the molecule at the expense of another in such a manner that a net amount of energy is set



free. In the usual anaerobic processes the energy transfers are of the types already described.

However, in aerobic metabolism the principal end products are carbon dioxide and water or hydrogen peroxide. Among bacterial species the most common aerobic terminal oxidations involve the reduced flavinenucleotides as illustrated previously or more frequently the cytochrome system. There is some evidence indicating that certain other metallo-enzymes may serve as terminal oxidases in certain bacteria, but the first two systems are probably much more common. Of these the cytochrome system is more frequently used for the terminal oxidation, for although the flavines are nearly always present they usually transfer electrons to cytochrome instead of to oxygen directly.

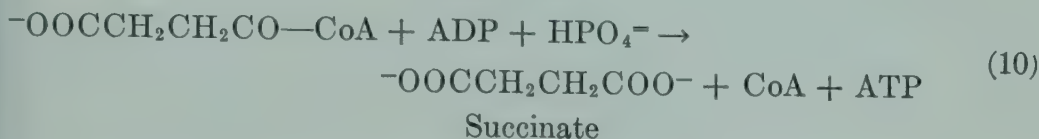
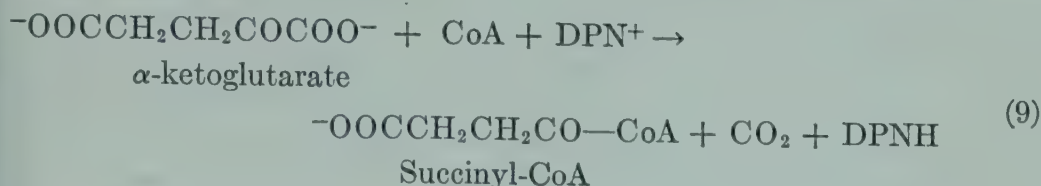
The cytochrome system is composed of a number of iron-porphyrin compounds whose general properties are rather well known. In these structures the iron undergoes reversible oxidation-reduction reactions. Furthermore, the iron-porphyrins themselves combine reversibly with various proteins, and such combinations have different oxidation-reduction potentials than do the dissociated iron-porphyrins. This latter fact contributes versatility to the activity and is partly responsible for the varied roles played by such compounds. In addition, since changes in acidity also alter the oxidation-reduction potentials, local intracellular pH differences may permit a still greater variety of action.

Several of these different iron-porphyrin-proteins called cytochromes are known. Some bacteria contain at least three, some one or two, and others as the clostridia contain none. In the last case reduced flavinadenine-dinucleotide transfers electrons directly to oxygen, but in the others the electrons are transferred to one of the cytochromes. The subsequent course of events involves the stepwise transfer of electrons to the other cytochromes and finally through the mediation of cytochrome oxidase (also an iron-porphyrin-protein) to molecular oxygen. These various stages involve energy transfers resulting finally in a maximum oxidation of carbon and hydrogen to carbon dioxide and water. In no step of this process beginning with  $\text{FADH}_2$  are phosphorus compounds known with certainty to be involved directly. The importance of the cytochrome systems is apparent when it is recalled that the oxidation of glucose to carbon dioxide and water yields ten times the energy obtained when fermented to lactic acid. Clearly then the competitive position of organisms is favorable when they are able both to accomplish the complete oxidation and to utilize the energy made available in this way.

A recently discovered type of energy transfer system is now known to be quite important in certain reactions at high levels of energy. A coenzyme called coenzyme A (CoA) is a key part of the general process. Of the several



examples known the oxidative decarboxylation of  $\alpha$ -ketoglutarate may be used to illustrate.



The enzymatic reaction (9) yields DPNH which can transfer the chemical energy residing in it by the mechanisms discussed in the sections on phosphorus in energy transfer. The succinyl-CoA formed at the same time is a substance showing a high free energy decrease on hydrolysis. However, a second reaction (10) is coupled to this process, instead of an unspecific hydrolysis, and the energy inherent in the succinyl-CoA is transferred to phosphate in the form of ATP as shown. Other known high energy transfers involving coenzyme A take place during the synthesis of citrate from pyruvate and oxaloacetate and during the metabolism of fatty acids. Thus this class of reactions has a quite general utility. It will be clear that such reactions as (9) yield a compound (succinyl-CoA) of the high energy type, but one in which phosphorus plays no direct part.

### OXIDATIVE ASSIMILATION

When a suitable material is added to a suspension of washed aerobic bacilli, it is metabolized, oxygen is consumed, and carbon dioxide is evolved. If no assimilable nitrogen is included in the medium, multiplication does not occur and yet there may be a gain in the dry weight of the organisms. To this phenomenon is applied the term *oxidative assimilation*. It is now known that part of the oxidized substrate is converted to water and carbon dioxide, and the remainder is incorporated into cellular structure apparently in the form of carbohydrates or as extracellular gums as in the case of *Rhizobium*. The specific anabolic reactions participating are presently unknown, so that only general information may be offered on the mechanism of oxidative assimilation.

The experimental basis for oxidative assimilation includes manometric data which reveal that when a limited amount of glucose, for example, is used as the substrate for washed *Escherichia coli* in phosphate buffer, the glucose completely disappears. Yet of the oxygen required for complete oxidation of the sugar only 60-65 per cent is actually taken up. Likewise

the carbon dioxide evolved is low in quantity, and since the respiratory quotient is 1 or less, part of the glucose vanishing from the medium must be retained by the cells. Although the glucose metabolized is not completely oxidized, a search for small organic extracellular waste products does not yield appreciable quantities. On the other hand, the dry weights of the organisms have risen somewhat, indicating storage of part of the substrate within the organisms themselves.

When carbon balances are run on washed *Escherichia coli* organisms, all of the added carbon may be accounted for in the unconsumed substrate, the organisms, and the carbon dioxide evolved. Of equal importance is the observation that the carbon of the glucose molecules oxidized appears both as carbon dioxide and as additional cellular carbon. The quantitative recovery data for the carbon balance experiments agree well within experimental error (Table 44).

This general type of behavior occurs with all the aerobic organisms studied and may indeed occur with many more substrates and organisms than not. Some substrates, however, may be oxidized and energy obtained therefrom but without the observation of simultaneous anabolic processes leading to the assimilation of carbon. In such cases it has been assumed that the oxidation reactions occur by a pathway incapable of yielding intermediates for the synthetic processes. Ordinarily substrates with which oxidative assimilation cannot be demonstrated are low molecular weight substances and show this anomalous behavior only for particular species. Lactic acid bacteria oxidize glucose but do not assimilate a significant amount of it. Instead they gain their carbon from amino acids which must be present to keep the organisms alive for more than a short period of time.

Multiplying bacteria must assimilate carbon in order to provide the new organic structure being formed. Studies similar to those illustrated above with resting cell suspensions have indicated similar general processes by which a given substrate is partly oxidized and partly converted to cellular constituents during multiplication. When nitrogen is present under these latter circumstances, amino acids, nucleic acids, and all other nitrogenous products as well as carbohydrate become ultimate goals of assimilated carbon.

Nitrogen assimilation resembles that of carbon somewhat except that nitrogen compounds are oxidized to a relatively lesser extent than is glucose by many bacterial species. Therefore, more nitrogen serves as a raw material for syntheses. Various nitrogen compounds may be utilized by many species and as is well known some organisms possess specific requirements. Such exacting species may require certain substances as general

nitrogen sources, or they may require individual compounds which are needed for particular metabolic purposes but which cannot be synthesized.

Nitrogen assimilation is not invariably an oxidative process since the nitrogenous raw materials assimilated need not always be oxidized. Nevertheless, in such cases oxidation of some other substrate is required for assimilation of the nitrogen compound. *Streptococcus faecalis* takes up glutamic acid but only while glucose is being oxidized or when some other similar energy source is available. In a sense, therefore, nitrogen assimila-

TABLE 44

*Comparative carbon balances in the oxidative assimilation of several substrates during the growth of Escherichia coli*

(Duration of experiment 4.5 hours)

SUBSTRATE, mg. C	SUCCINATE	FUMARATE	LACTATE	PYRUVATE	GLYCEROL
Initial substrate-C.....	5.51	3.14	3.31	4.45	5.40
Cell-C after assimilation.....	0.55	0.44	0.37	0.51	0.72
Cell-C before assimilation.....	0.35	0.21	0.29	0.20	0.51
C stores.....	0.20	0.23	0.08	0.31	0.21
Supernatant-C at end of experiment...	5.04	2.57	3.19	3.76	5.15
CO <sub>2</sub> -C.....	0.29	0.30	0.12	0.39	0.10
Total recovered.....	5.53	3.10	3.40	4.46	5.46
Total recovered, per cent.....	100.3	98.7	102.7	100.2	101.1
μl CO <sub>2</sub> produced.....	536	534	214	724	182
μl O <sub>2</sub> consumed.....	380	274	222	394	266
R.Q. observed.....	1.41	1.95	0.97	1.84	0.70
C stored/CO <sub>2</sub> -C.....	0.7	0.8	0.7	0.8	2.1

(Adapted from Siegel & Clifton, 1950.)

tion also indirectly becomes an oxidative process. It has been suggested that the energy coupling may involve active transport across cell barriers, endergonic utilization of the nitrogenous compounds intracellularly, or both.

The assimilation of carbon during oxidation of a substrate has been demonstrated in algae and in fermentations by yeasts. Consequently, oxidative assimilation is not limited to bacteria or to aerobic systems so that the phenomenon is of rather general occurrence. In the anaerobic cases the fraction of substrate converted into cellular carbon or nitrogen can be considerable.

Clearly, oxidative processes are required for all anabolic syntheses, but the literature is not always objective regarding the relative importance



of assimilation and dissimilation of particular nutrients. The phenomenon of oxidative assimilation is an example of how oxidation of a substrate may serve a dual purpose as a source both of energy and of intermediate metabolites required for synthetic processes. A further finding that demonstrates the importance of the assimilatory function of oxidation, in contrast to the purely energy yielding function, is the observation that oxidizable substrates of similar carbon content but dissimilar energy content can supply equal quantities of assimilated carbon during oxidative assimilation. *Escherichia coli* assimilates as much carbon from pyruvate as from lactate and as much from fumarate as from succinate in spite of the fact that pyruvate has a lesser free energy content than lactate and fumarate than succinate. Thus the assimilation is at least to a certain extent more dependent on the chemical nature than on the free energy content of the oxidized substrate.

With organisms that are metabolizing but not growing, attention has been centered mainly on problems of energetics, and the importance of catabolic reactions used purely for the purpose of supplying building units for synthetic purposes has not always been kept in mind. It is well known that metabolizing organisms carry out a great deal of synthesis merely to replace materials deteriorating during the normal life processes. Even though body weight does not change and composition is essentially constant, these exchanges occur, continually replacing enzymes and the like. True, there is no net assimilation but just as truly many of the same reactions must be operating. Presumably if this anabolism is prevented by starvation, the organism will "run down" by endogenous catabolism and die.

Recognizing that anabolism in general and oxidative assimilation in particular are important, one should not assume them to be the sole functions served by energy consuming processes. On the other hand, energy occasionally has been regarded as incidental to assimilation, but such an extreme viewpoint is forced to acknowledge that many if not most of the assimilative syntheses must be coupled to energy transferring steps. The most reasonable attitude would seem to regard the synthetic and energy yielding processes as dependent on each other and both important but filling roles whose relative importances cannot be compared because the fundamental purposes of the processes are different.

The facultative autotroph *Pseudomonas saccharophila* may be used to illustrate this close inter-relationship. Tracer studies have shown that this organism almost completely oxidizes the carboxyl group of lactate to carbon dioxide. On the other hand, only 20-30% of each of the other two carbon atoms is so oxidized. Hence most of the carbon in positions 2 and 3 is assimilated into cellular material. It would appear, therefore, that

part of the energy from the oxidized portions of the lactate ion is utilized in syntheses by means of coupling to high energy intermediates like ATP. Another fraction of the energy may maintain the thermodynamically unstable components of the organism. This suggestion leads logically to a consideration of the subject of endogenous catabolism.

### ENDOGENOUS CATABOLISM

Among higher animals it is readily apparent that bodily processes requiring energy do not cease during starvation. Muscular activity is possible in humans after many days without food, and the energy thus utilized is withdrawn from compounds stored within the body. When other organisms including bacteria are cut off from their food supplies metabolism goes on at a diminished level with the oxidation of various materials previously accumulated. This activity is called *endogenous metabolism* (or *catabolism*) thus denoting the location of the substances supplying the energy.

Several questions concerning endogenous metabolism may be asked. Is such activity necessary for the preservation of life? Does this activity continue even in the presence of a plentiful source of nutrients? What are the cellular substrates attacked? How is this metabolism carried out? While in general clear-cut answers cannot be given to these questions it will be instructive to consider some of the more widely circulated of the pertinent ideas and hypotheses.

Owing in part to uncertainty concerning the nature of life it is difficult to know whether endogenous respiration is necessary for the maintenance of viability. Life is usually thought to have ceased when various normal processes can no longer be observed. One of the more useful criteria is that of respiration, and when the evidences of respiration have disappeared the organism is said to be dead. For example, washed bacteria continue to respire endogenously when held in a non-nutrient medium; however, with time the uptake of oxygen and the evolution of carbon dioxide slowly decline. If samples are withdrawn during the period of starvation and placed under conditions suitable for growth and multiplication, the number of viable bacteria is observed to steadily diminish as starvation proceeds. Finally no viable organisms are found, and the bacteria are said to have died. It has seemed logical in the past, therefore, to assume that some sort of respiration is necessary to sustain viability.

A new and complicating fact raises doubts about this last assumption. Bacteria are very susceptible to the highly toxic mercuric ion and are readily "killed" by mercuric chloride. However, it has been possible to remove the metal from these "dead" organisms, and they have then resembled ordinary, untreated bacilli and been capable of respiration and multiplication. As a result of this finding is one justified in stating that



endogenous respiration is necessary for life? It would seem not, for though the bacteria treated with mercury have no *detectable* respiration they cannot be classified as dead because they can be so treated as to allow them to function in a typical fashion. Since we are attempting throughout this consideration to interpret negative data, caution must be observed. Clearly the failure to observe respiration may be merely a quantitative matter although this does not seem likely. In addition no thorough search has been made for evidence of other kinds of metabolic activity other than the obvious uptake of oxygen or evolution of carbon dioxide.

If endogenous catabolism is not essential for some life process, its function is still unexplained. Various suggestions have been made including the purely descriptive concept of wear and tear. Mechanical activity has been implicated in the destruction of critical components of the organism. Presumably energy for this process of repair can be obtained from structural materials in order to keep the organism alive in the absence of an external food source. However, it is difficult to visualize how an individual or bacterium at rest suffers any mechanical wear and tear.

As a result of this objection a somewhat similar theory arose but one based on notions of chemical wear and tear. Under normal conditions the organism employs oxidation of foodstuffs for purposes of securing energy. When the ordinary substrates are not available, it has been postulated that essential compounds, e.g. enzymes, are oxidized and that a reduction of a fraction of these key materials must be carried out to keep the individual alive. Energy for the reduction is, of course, assumed to come from the metabolism of other parts of the organism. It is here implicit that such a net oxidative deterioration would not occur when normal foodstuffs are available nor when oxygen is absent. Neither notion appears to be true in fact. Actually there are experimental data indicating that endogenous catabolism continues in at least some organisms while normal foods are being metabolized and in yeast may even be increased in the presence of an external source of oxidizable substrate. An experiment indicating the occurrence of endogenous respiration in a bacterial species in the presence of an external source of glucose is discussed in Figure 78.

In the light of these facts another interpretation states that endogenous respiration merely represents degradative processes that are continually occurring. In starved cells this activity is readily observed, but it takes place under ordinary conditions also. No useful function is assumed for the endogenous catabolism, and the general result would be a valueless series of spontaneous reactions. For as long as an enzyme can contact its specific substrate catalytic activity goes on and as long as an organism is within the biokinetic temperature range some protein denaturation, no matter how slow, must be expected. This theory is one of the type that



can be proved only by exhaustive study of numerous cases and can be disproved by a single conflicting observation.

Another possibility for the spontaneous breakdown of cellular constituents during starvation is based on the fact that the synthetic reactions of organisms are usually reversible and the removal of the normal exogenous substrates may well allow a reversal of the metabolic reactions. In this way the cellular material would be degraded to compounds of lower energy content. On the other hand, starving animals are capable of muscular exertion and initially healthy motile bacteria will remain motile for a time in a properly buffered isotonic solution free of nutrients. Evidently then endogenous catabolism can yield useful energy under certain conditions. Interesting illustrations of this situation are the studies made of the carbon dioxide fixing capacity of the autotroph *Thiobacillus thiooxidans* when respiring endogenously.

Resting cell suspensions of this organism were exposed to sulfur in the absence of carbon dioxide. Phosphate uptake occurred upon the oxidation of the sulfur, but no growth was possible in the absence of a carbon source. Subsequently when the organisms were freed of inorganic sulfur and carbon dioxide was introduced into the culture, the carbon dioxide was fixed and phosphate simultaneously released. In this situation it is obvious that the assimilation of the carbon dioxide is drawing upon an endogenous substrate for energy since the exogenous energy source has been withdrawn.

An interesting type of endogenous activity is sporogenesis. The formation of bacterial spores is associated with the synthesis of new organic compounds including antigenically specific spore proteins and carbohydrates. If the vegetative forms of an aerobic spore-forming bacterial species are removed from a culture at the proper stage of development and placed in aerated distilled water, spore formation occurs. In this situation it is evident that the raw materials for the synthesis of spore-specific compounds and the energy for these syntheses are derived solely from endogenous metabolic activity.

Many of the compounds involved in metabolism have higher free energies than the original substances from which they were formed. Enzymes, for example, should tend to hydrolyze when in an aqueous medium unless energy is available to keep the reaction on the side of synthesis. *Energy of maintenance*, the term applied to the energy needed for maintaining the status quo, may have its source in endogenous respiration.

When a bacterium metabolizes exogenous substrates it accumulates critical substances first and later stores reserves which often can be recognized cytologically as inclusion bodies. During starvation the reserves disappear, but the critical components are preserved until the stress becomes too great and the organism dies. Thermodynamic concepts suggest

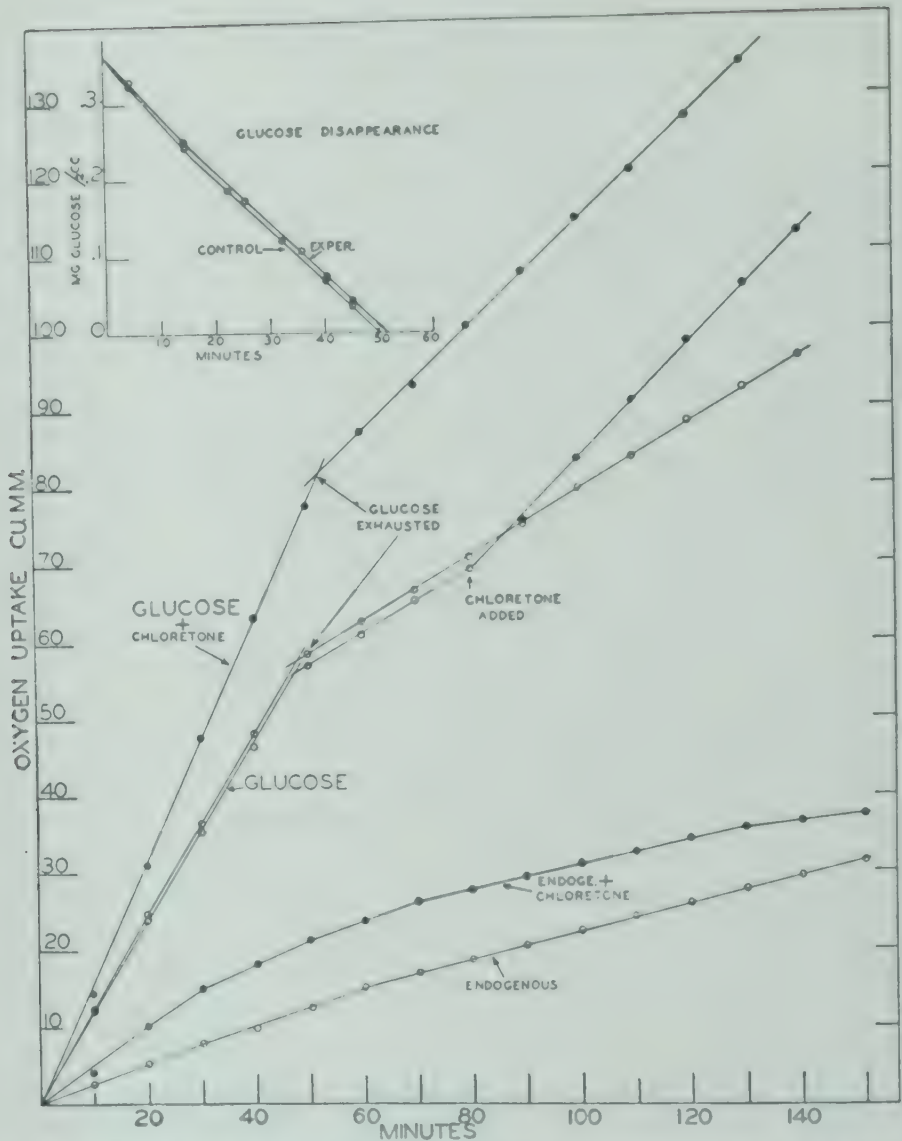


FIG. 78. The effect of 0.003 M chloretone on the oxygen uptake of *Achromobacter fisheri* at 19°C. with and without glucose.

(From McElroy, 1944).

This experiment represents an attempt to determine the influence of the dissimilation of an exogenous substrate (glucose) on endogenous respiration. Oxygen uptake appears to be stimulated by the chloretone. This stimulation has been interpreted as due to the stimulation of endogenous respiration concurrent with oxidation of an external source of glucose. Such a conclusion is based on the exhaustion of the supply of glucose at the same time in the absence of chloretone and in systems containing chloretone. Obviously, if chloretone were stimulating the uptake of the glucose and not endogenous respiration the glucose should disappear more rapidly in the presence than in the absence of chloretone.

While the general approach to the study of endogenous catabolism in the presence of an external oxidizable substrate attempted in the experiment is valid, any conclusions drawn from the particular data must be accepted with reservations. It

that compounds of low energy content ought to be synthesized early and be retained the longest. Evolution should have selected organisms capable of withstanding starvation stresses, and these successful organisms ought to have critical substances possessing relatively low free energies in order to maintain them for a maximum time. Unfortunately, we know of no data collected with the intention of testing this possibility.

Attractive as is the concept of energy of maintenance it is not yet established as fact, and there are even data which challenge it. One would anticipate that a culture respiring endogenously at a given rate should slowly lose the substance of its individuals. If low concentrations of external substrate were made available it ought to be possible to balance the loss of energy and materials from the organisms. Therefore, at some value the status quo should be maintained indefinitely without growth. At higher concentrations of nutrients growth should occur. In Figure 79a the amount of growth of *Escherichia coli* is plotted against substrate concentration. Extrapolation of the data to the origin of the graph indicates that growth actually takes place at all substrate concentrations. If this is true no glucose is needed to provide sustaining energy, otherwise growth should not take place below some limiting concentration of nutrient. Hence, the existence of energy of maintenance has been challenged. One cannot accept

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will be instructive for the student to consider some objections which can be raised to the above data.

In the first place the data indicate that chloretone does not stimulate the disappearance of glucose from the medium while it does stimulate oxygen uptake. However, other conceivable processes than stimulation of endogenous respiration may account for this observation. In this connection it is important to note that the amount of oxygen consumed up to the point of complete disappearance of glucose is but a small fraction of that needed for the complete oxidation of the 0.36 mg. of glucose originally present. The oxygen consumed corresponds to the amount expected if glucose utilization leads to formation of 2-ketogluconate or some equivalent compound. Since no data on carbon dioxide evolution are presented it is not possible to estimate the quantity of glucose actually oxidized to carbon dioxide and water. In any event the chloretone may be influencing reactions subsequent to glucose uptake in addition to those reactions specifically involved in the endogenous catabolism of resting cell suspensions not initially exposed to glucose.

Nor is it clear from the data presented that the curves in the presence of glucose reveal any endogenous catabolic activity whatever, since materials derived directly from glucose apparently are present in large amounts and may readily account for all of the oxygen consumed. Had the data been taken for a long period *until the glucose was completely oxidized*, some answer might be made to this objection. Hence, it would seem that the only possible basis for the interpretation as originally made would require that endogenous catabolism be defined as the respiration of any intracellular substance regardless of its metabolic origin, nature, or position in the metabolic pathway. Such a broad definition does not appear to be intended by most investigators.



this kind of data at face value, however. Data obtained at much lower concentrations of nutrient (Figure 38, Chapter 8) may be in conflict with those obtained in the experiment of Figure 79a for it may not actually be per-

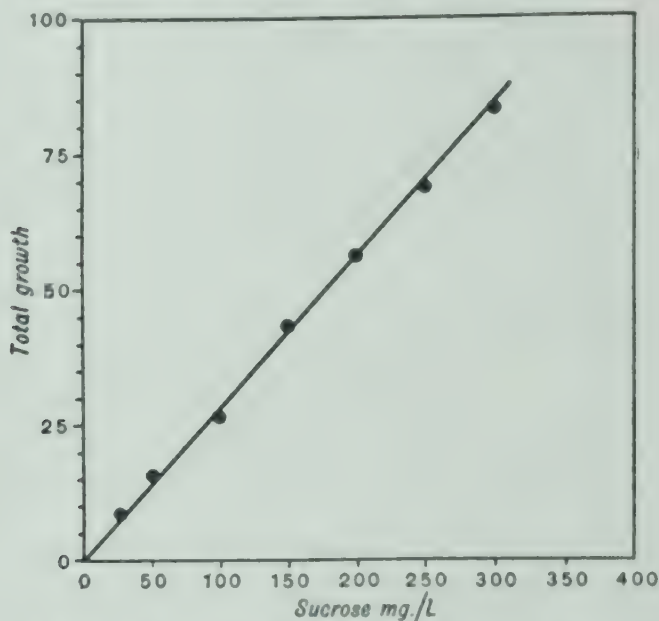


FIG. 79a. Total growth of culture of *Bacillus subtilis* in synthetic medium as a function of the concentration of sucrose.

(From Monod, 1942)

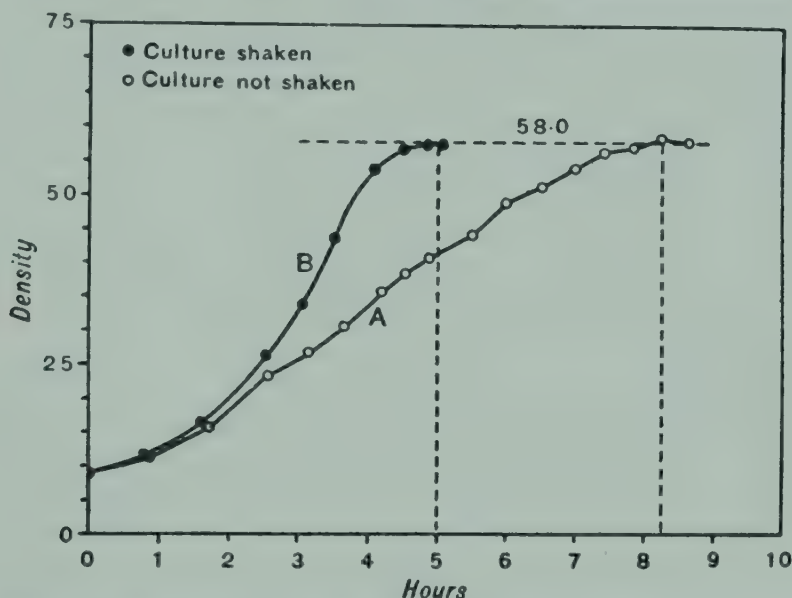
The extrapolation of the data to the origin would indicate that *all* of the sucrose utilized is involved in the growth process and none is consumed for energy of maintenance. But there is no reason *a priori* for believing that any external food source must serve as the oxidizable substrate for energy of maintenance. Energy of maintenance may be immediately available only from an endogenous substrate by way of endogenous respiration. In the above growth experiment this possibility is not taken into account, and therefore the data cannot be seriously considered to have disposed of the problem of energy of maintenance. In a growth experiment the sucrose assimilated simultaneously with growth provides carbon for the synthesis of endogenous substrate from which in turn the energy of maintenance might be derived.

If sucrose yields energy for assimilatory processes and for the net synthesis of products A, B, C, D, E collectively identified as growth and if E alone or in combination with any other product or products functions in supplying energy of maintenance, then the mere measurement of growth in relation to sucrose utilization cannot reveal the existence of energy of maintenance.

missible to extrapolate the data to yield a curve intersecting the origin. It has been pointed out in the section on the influence of solid surface on growth as well as in the chapter on growth that there is some evidence for the existence of minimal concentrations of nutrients below which growth does not take place. An extensive reinvestigation of growth in dilute solutions of nutrients seems desirable. Furthermore, endogenous respiration

clearly involves the elimination of degradation products into the medium resulting in a loss in weight of the individual organism. If, as suggested previously, endogenous catabolism continues even when an exogenous substrate is being utilized, it is difficult to conceive how the merest trace of nutrients can possibly result in net synthesis, that is, promote growth.

Another approach to the problem is revealed in Figure 79b. Assuming that endogenous catabolism continues throughout the course of the experiment then the same total growth should not be reached by two clones of the same pure line growing at different rates in the same kind of medium



79b. Growth of two cultures of *Escherichia coli* on synthetic medium plus ammonium lactate. A at rest, B shaken.

(From Monod, 1942)

and at the same temperature. Stated in another way, the extra energy diverted into endogenous processes by the slowly growing culture because it takes longer to reach a peak population should result in a reduced total population. This expectation was not realized in the experiment designed to test this notion and recorded in Figure 79b in which a difference in growth rate was achieved by varying the degree of aeration of otherwise similar *Escherichia coli* cultures. Again the problem of the existence of energy of maintenance has not been answered but rather made more baffling. In the logic employed in the experiment it necessarily is implied that the substrate providing energy is the only factor limiting total growth. In the particular experiment cited this assumption has not yet been justified by data and thus places in question any interpretation of the meaning of the experiment. Most important it has not been established that the metabolic

processes provide assimilated substance of the same energy content in the two different cultures, nor is it known whether the energy source is completely consumed in both cases. Furthermore, to interpret the experiment quoted as proving the non-existence of energy of maintenance one would also need to accept another and unproven hypothesis, namely that energy of maintenance, if it existed, would be the same for both the anaerobic and aerobic states of a given organism.

What is the nature of the substrates subjected to endogenous respiration? A complete answer promises to be quite difficult to reach because of the complexity of organisms and because many if not all of the various components present undergo exchanges. Quantitative composition data provide the only answer, and the techniques necessary for obtaining these data are still largely unavailable. Actually only clues have been obtained, but these at least suggest the direction of the ultimate search. Suggestive of the nature of the substrates, and metabolic paths of utilization would be knowledge of the kinds and relative quantities of excretory products resulting from endogenous respiration of bacteria under different conditions. Unfortunately, except for  $\text{CO}_2$  evolution practically no data of this kind are available.

Organisms respiring aerobically consume oxygen and yield carbon dioxide. The relative quantities of these two gases (respiratory quotient or R.Q.) bear a relationship to the general nature of the material being metabolized. When the endogenous respiration of bacteria is followed by means of respiratory quotients it is common to observe a gradual change over an extended period of time. This shift is usually to lower values and is taken to indicate a change in the nature of the cellular materials being metabolized to compounds of a greater state of reduction.

Cytological evidence for a change with time in the nature of the substrates endogenously catabolized has already been cited in the case of the loss of the gram positive reaction of starving *Bacillus cereus* associated with a reduced respiratory quotient. With *Mycobacterium tuberculosis* the respiratory quotient is 0.85 immediately after the organisms are placed in the starvation medium. After 24 hours the quotient falls to 0.78 and after seven days to 0.72. Presumably, the endogenous catabolism of this latter organism involves stored lipids whose composition changes somewhat as the starvation proceeds. Viability of some tubercle bacilli in cultures has been observed after starvation of up to 55 days although most other species cannot survive such a prolonged period of endogenous activity within the optimum temperature range.

*Pseudomonas aeruginosa* may be taken as an example of species whose endogenous catabolism appears to involve storage products other than lipids. In this organism respiratory quotients of 1.1 have been reported



which strongly suggest that highly oxidized materials are involved. Presumably, these substrates are at least partly carbohydrate in nature, perhaps containing carboxyl groups in order to account for the R.Q. slightly higher than 1.

The R.Q. of *Thiobacillus thiooxidans* is close to 1.0 and indicates the probable storage of a carbohydrate type of reserve food by this autotroph. It proves that the nature of the endogenous respiration of autotrophic bacteria is like that of heterotrophic species and involves the oxidation of organic compounds.

Evidently there are species differences among bacteria regarding the nature of the food reserves, some storing lipids, others carbohydrates, and still others probably nucleic acid. In addition there are differences in the quantities stored with these variations dependent upon the strain, physiological age, nutrients, and cultural conditions. Such fluctuations necessitate careful control in the preparation of resting cell suspensions for studies of the rates and mechanisms of respiration and fermentation.

As difficult to answer as the preceding questions is the final one concerning the mechanism of endogenous catabolism. One might assume as a working hypothesis that endogenous respiration is a reversal of the mechanisms employed in the synthesis of the storage products which are later dissimilated during starvation. Many of the individual reactions are known to be reversible and at least part of the same pathway should be usable in the reverse direction.

Since some of the typical chemical inhibitors of respiration act on the endogenous process, known kinds of reactions could be affected. Furthermore, the metabolic utilization of a normal external substrate is reported to stop endogenous respiration for a number of bacterial species. This interference indicates a close relationship, perhaps through utilization of some of the same metabolic reactions.

On the other hand, other bacteria (see Figure 78), *Streptomyces coelicolor*, and yeast species are known to respire endogenously while an exogenous substrate is being metabolized. One might, therefore, anticipate separate pathways for endogenous and exogenous respirations. However, this need not be the case if certain assumptions can be justified. If the quantities of the enzymes involved in the common portions of the metabolic route are quite high, then catalytic agents sufficient for both types of process will be available. Hence both can proceed simultaneously by the same reactions, providing the rate of endogenous metabolism is limited at some point preceding the common pathway. This hypothesis has not been examined experimentally and should be viewed critically because of the severe conditions it would impose on the structural organization of the cell.

Among the bacteria the rates of endogenous respiration vary widely as

indicated by a  $Q_{O_2}$  of 0.3 at 32°C. for endospores of *Bacillus subtilis* and values of 18 for vegetative forms of some saprophytic species,  $Q_{O_2}$  denoting the number of microliters of oxygen consumed per hour by bacilli having a dry weight of one mg. In general the acid-fast bacteria have among the highest and most variable rates of endogenous oxygen consumption. This property has interfered a good deal with studies of the metabolism of these aerobic organisms.

During investigations of the metabolism of exogenous substrates, knowledge of the rate of endogenous respiration is important because quantitative manometric results are very valuable and in many cases indispensable for understanding metabolic pathways. It is necessary, therefore, to measure endogenous rates as well as the rates of total respiration. Furthermore, one must ascertain whether the endogenous rate is maintained in the presence of the added substrate in order to correct for it legitimately. It happens that very high values of endogenous oxygen uptake ( $Q_{O_2}$ ) may nearly disappear during exogenous respiration, so that suitable corrections can be made only when this change is known. Several erroneous conclusions have appeared in the literature when this possibility has been overlooked.

Since endogenous activity is stimulated in at least the case of bakers' yeast by exogenous catabolism, an opposite effect may be important. It is not yet possible to predict how universal such increases may be, nor are simple techniques available to study the question. Because most of the conclusions drawn without recognition of the opportunity for an increase in endogenous respiration have been verified by means of other approaches, this unjustified assumption may not lead to the invalidation of much of the work being done.

Inasmuch as endogenous processes now are known to be sharply reduced in a variety of cases in the presence of external nutrients, attention has been devoted toward reducing the endogenous activity as much as possible without killing the cells. Frequently this may be achieved by preliminary aeration of a resting cell suspension without added substrate. Prolonged storage in the cold accomplishes the same purpose as aeration in that both allow the occurrence of endogenous catabolism until it has slowed down. Still other schemes depend upon the harvesting of the organisms early in the growth cycle before large quantities of reserve products have been stored and on growing them in media low in carbohydrate and high in nitrogen compounds. In these ways endogenous activity is kept low by limiting the amount of stored utilizable substrate.

#### INTERMEDIATE METABOLISM

The field of investigation of intermediate metabolism is the study of the mechanism of metabolism. It has developed into a search for inter-



mediate compounds, the reactions undergone by these substances, and the modes of transfer of energy. The subject, both complex and extensive, has supplied ample opportunity for numerous outstanding investigators to display their talents. Work has progressed so rapidly that only a general summary can be undertaken. Therefore, attention will be devoted to the overall results of investigations in the field of intermediate metabolism and to methods of investigation of broad application. For convenience it is desirable to separate the discussion of intermediate metabolism into various phases. Since all these phases are closely interrelated, it is necessary to remain aware of the interdependence.

#### METHODS OF STUDY

The following methods of investigation include the more general procedures employed in the study of intermediate metabolism. In relatively few cases will a single type of study be sufficient to limit the possibilities to one in the complex systems under examination. Therefore, it is common practice to combine two or more approaches for the solution of most problems. As might be expected, the best combination will depend upon the needs of the particular problem, the skills of the investigator, and the equipment available.

#### *Manometric Techniques*

A variety of manometric apparatus is now available for the study of problems in metabolism and which may be used in many ways to obtain data of many different kinds. For complete discussions of the apparatus and the details of use, reference is made to the books by Dixon (1951) and by Umbreit, Burris, and Stauffer (1949).

In general the manometric methods depend upon gas exchanges, usually between a gas phase and a solution phase. Nearly any reaction involving a gas as either a reactant or a product may be studied manometrically. In practice, measurements are limited to reactions occurring at ordinary temperatures and pressures and at appreciable rates. When more than one gas is exchanged the situation becomes complex but may be dealt with under certain conditions. Especial attention has been devoted to the study of systems involving exchanges of both oxygen and carbon dioxide since these two gases are so important in respiratory processes.

When individual reactions like that below



are under investigation, the theory, apparatus, and procedure are comparatively simple. In a great many such *in vitro* cases much has been learned of the properties of enzymes and of their mechanism of action. Using more complex materials such as tissue slices, suspensions of bacteria, or cellular



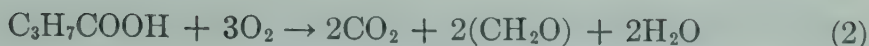
extracts it has been possible to place many of the individual reactions in their proper places in overall processes. Extensive contributions of both sorts have resulted from the use of manometric techniques, and new data so obtained are appearing at an accelerating rate.

For a number of purposes it is useful to compare the carbon dioxide evolved to the oxygen taken up. The ratio of these two quantities, called the *respiratory quotient* (R.Q.) is characteristic of the substance being oxidized. As was pointed out earlier the general nature of substrates may be inferred from the respiratory quotient since carbohydrate, protein and fat have values respectively of 1.0, 0.9, and 0.8. Thus it becomes possible to suggest the category into which a natural substrate falls. Sometimes, however, there are metabolic complications which shift the observed value of the respiratory quotient from its normal range. Although this effect complicates the identification, it indicates the existence of peculiarities of metabolism and thereby points out new research problems.

Manometric procedures are widely used when attempting to identify metabolic intermediates. Postulated compounds are added to tissue preparations, suspensions of organisms, or cell extracts. If the enzyme systems present are capable of utilizing any given substance, this material disappears from the medium. Under aerobic conditions oxygen will be consumed in excess of the endogenous rate, and if respiration is complete carbon dioxide will appear.

When oxidation of the substrate is complete, the quantities of oxygen and carbon dioxide exchanged may be predicted from the nature and amount of substrate disappearing. The respiratory quotient will then be that obtained by an ordinary, complete chemical oxidation. Table 45 indicates that such data may be quite characteristic. When measurements are made with a known substrate, butyric acid for example, and an unexpected respiratory quotient observed, as 0.67, then one is led to suspect complicating side reactions. Two general possibilities are open. First some other reaction may be overshadowing that of the butyrate oxidation. This possibility may be tested by comparing the disappearance of butyrate with the rates of exchange of the two gases. If oxygen and butyrate disappear in molar quantities approximately in the ratio of five to one, as expected from the balanced, complete reaction, then this substrate reaction is the preponderant one.

As the remaining alternative, part of the butyrate is being utilized but is being only incompletely oxidized. Thus oxygen is consumed but part of it is retained in non-gaseous products either in the cell or medium, and the carbon dioxide output is correspondingly diminished. This general idea may be expressed with the hypothetical reaction (2) using butyric acid as a substrate.



( $\text{CH}_2\text{O}$ ) is taken to represent the material formed during this oxidative assimilation. The respiratory quotient is seen to be 0.67 in this instance.

A great variety of microorganisms form an acid of some kind as a product of their metabolism. In many such cases the quantity of acid may be related to growth or to total metabolic activity, and the measurement of the acid thus supplies useful data. For this estimation, manometric procedures are widely employed since in the presence of the acid the evolution of carbon dioxide from bicarbonate solutions may be followed easily. This particular use is but one of the many which might be listed. Yet an extended enumeration of applications would serve no useful purpose since the same principle is involved in all.

TABLE 45

*Sample reactions of and the resulting respiratory quotients of acetic acid, pyruvic acid, and butyric acid*

OVERALL OXIDATION REACTION	RESPIRATORY QUOTIENT
$\text{CH}_3\text{—COOH} + 2\text{O}_2 \rightarrow 2\text{CO}_2 + 2\text{H}_2\text{O} \dots\dots\dots$	1.0
$2\text{CH}_3\text{—COCOOH} + 5\text{O}_2 \rightarrow 6\text{CO}_2 + 4\text{H}_2\text{O} \dots\dots\dots$	1.2
$\text{CH}_3\text{—CH}_2\text{—CH}_2\text{—COOH} + 5\text{O}_2 \rightarrow 4\text{CO}_2 + 4\text{H}_2\text{O} \dots\dots$	0.8

### *Resting Cell Studies*

Much metabolic work involves the use of *resting cells* which are ordinary organisms not multiplying. The manometric methods just described are most commonly applied in such investigations, but almost every procedure employed in studies of metabolism is used at least part of the time with resting cells.

Although not growing, organisms nevertheless act metabolically. Processes of respiration (or fermentation) may thus be conveniently separated from the complicating processes of growth when resting cells are studied. Resting cells offer other advantages as well, inasmuch as their activity is at rates high enough to be measurable within short periods of time, they are readily standardized, can be washed free of growth factors present in the culture medium, and they need not be absolutely pure strains. In this latter connection contamination with small quantities of other kinds of organisms does no harm because there is no opportunity for the contaminant to outgrow the type under study. When changes are intimately related to multiplication, e.g., the increase in desoxypentosenucleic acid associated with cell duplication, resting cells obviously cannot be used.



However, many if not most of the reactions of metabolism occur with resting organisms and are readily studied therein.

In the determination of respiratory quotients as a guide to the nature or the fate of a substrate, multiplication of the organisms complicates the analysis because much of the substrate may be converted into new cellular material. Even though oxidative assimilation can occur when a substrate is added to resting cells, the incorporation of substrate into cellular material is not usually so great as during growth.

Of more value is information that a compound can be utilized by a particular species. When resting bacteria act on a substance, it is assumed that these organisms must have a mechanism for handling that substance. Such information serves as a starting point in the studies of metabolic mechanisms.

Negative results in such utilization studies should not be taken as evidence that a given material cannot be metabolized by the organisms in question. A number of cases are known in which various species do not oxidize a compound added to a cell suspension but which actually synthesize the compound and do oxidize it when it is inside the cells. In these instances the lack of utilization of the exogenous material is attributed to impermeability with respect to the particular substrate. This type of situation is especially common among bacteria and has led to erroneous interpretations when it has not been taken into account.

As a rule resting suspensions of bacteria should be used when investigating the utilization of suspected intermediates. A slow disappearance of substrate may go unnoticed in the high general level of activity of growing cultures. Moreover, negative findings may be recorded even when the substance is a metabolite and actually does penetrate the organism. This type of difficulty may be encountered when the level of metabolic activity is high, so that the enzymic reactions expected to make use of the exogenous metabolite are acting fully on metabolite of endogenous origin. Both of these complications can be avoided by maintaining metabolism at a low level in the absence of the added material. For this purpose resting cells are treated so as to deplete them of endogenous sources of intermediates and substrates before the addition of an exogenous source of test substrate.

The pyruvate oxidation factor for *Streptococcus faecalis* may be cited as an example of work which can be done with resting cells in the discovery of new growth factors or metabolites. Cultures of strain 10C1 were harvested, washed, and tested manometrically for their ability to oxidize pyruvate. When the suspension of resting cells was prepared from bacteria grown on a medium containing the proper constituents, pyruvate disappeared at a low rate. However, the addition of a yeast extract to such a



preparation produced a ten-fold increase in the rate. The various known cofactors had no such effect indicating that an unknown factor in the yeast extract was involved in the oxidation of pyruvate. Various experiments all employing resting cells indicate that the organisms absorb and store the cofactor, thus suggesting that the unknown substance is a coenzyme. Purification of the material was undertaken once again using resting cells, this time to assay the fractions obtained from various sources of the pyruvate oxidation factor.

### *Selective Destruction and Replacement of Metabolites*

When a required metabolite is synthesized and stored by a species in quantities sufficient for the needs of the organism, the mere addition of that metabolite to a resting cell suspension, and a study of respiratory quotients will not reveal the requirement for or the utilization of the metabolite. Under such circumstances another approach is needed. One that may be employed in certain cases involves the selective elimination of the factor from resting cell suspensions and a study of the effects produced by re-addition of the substance to the deficient preparation. Either a natural source or purified preparations of the metabolite may be employed as replacement material.

Three different types of problems may be handled by the technique of selective destruction and replacement: verification of a suspected metabolic role of a compound of known properties in a particular species, discovery of new growth factors or metabolites, determination of the metabolic reactions in which a factor participates.

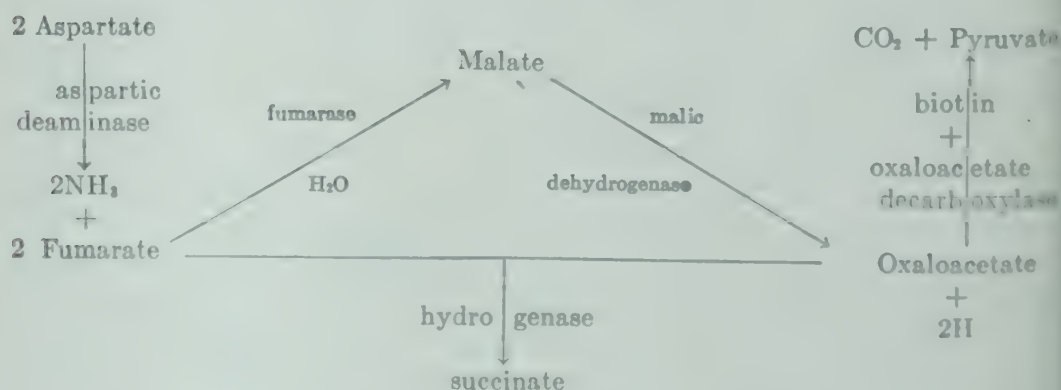
The first objective is achieved by observation of the restitution of normal metabolism upon addition of the factor to organisms previously rendered deficient. Discovery of new factors may be accomplished by various treatments aimed at disturbing the normal metabolism of organisms and attempting to reestablish normal activity by addition of natural products, such as vegetable juices, yeast extract, or fractions isolated from normal organisms of heterologous species or even from the homologous test species itself. Once restoration has been observed with some natural product the cells rendered artificially deficient may be employed as assay organisms in order to follow methods for the isolation and characterization of the factor.

Since the mere recovery of metabolism does not differentiate between new and well-known factors in complex natural mixtures time may be saved by adding back any known compounds whose destruction might result from the particular treatment used. In this way the more probable, known substances may be checked for their replacement value before a laborious isolation of an unknown substance is undertaken.

Once a factor is known to function in an organism it may be possible to determine the reactions in which it is involved. Deficient resting cells prepared by specifically destroying the factor of interest are added to various metabolites. Some of these compounds may be altered by means of reactions lying ahead of or beyond the break in a metabolic chain of reactions produced by destruction of the factor in question. The metabolites not acted upon by the deficient cells either do not penetrate the cells or their role involves a stage located preceding the metabolic block. If addition of the factor in question allows utilization of one of these previously unaffected compounds, then the compound clearly must penetrate the organism and participate in the reaction chain blocked by lack of the factor. Either from previous knowledge of the sequence of reactions or from information of this sequence obtained by juggling reactions and inhibitors, the exact site of action of the factor may be located.

These general principles may be illustrated with the elegant work that was done on the function of biotin in *Escherichia coli*. Originally it was observed that the bacilli harvested from non-synthetic media rapidly lost the capacity to metabolize aspartic acid. The activity was restored by addition of a mixture of B vitamins. Single addition of the members of the vitamin mixture revealed that biotin was the active factor and that it could not be replaced by any or all of the other known vitamins in the original mixture. Biotin is unstable in acid solution, and deficient cells could be obtained most readily by taking advantage of this fact. Therefore, bacteria were grown, harvested, and then held for a short time at pH 4. Much of the biotin synthesized by *Escherichia coli* is thus destroyed without otherwise greatly affecting the metabolism of aspartic acid.

With these acid-treated bacilli the postulated breakdown products of aspartate were studied. It was possible to implicate oxaloacetate in the reaction controlled by biotin. Once this was known other experiments



demonstrated that oxaloacetate itself was not part of the system but that a derivative of it, possibly a phosphorylation product, was converted to carbon dioxide and pyruvate. Biotin proved to be necessary for this decarboxylation.

This particular investigation showed that biotin is an essential component in the metabolism of *Escherichia coli* where it serves in the decarboxylation of a derivative of oxaloacetate. The reactions immediately preceding this step were also identified during the study of the role of biotin( see formula at bottom of facing page).

### *Inhibition and Accumulation*

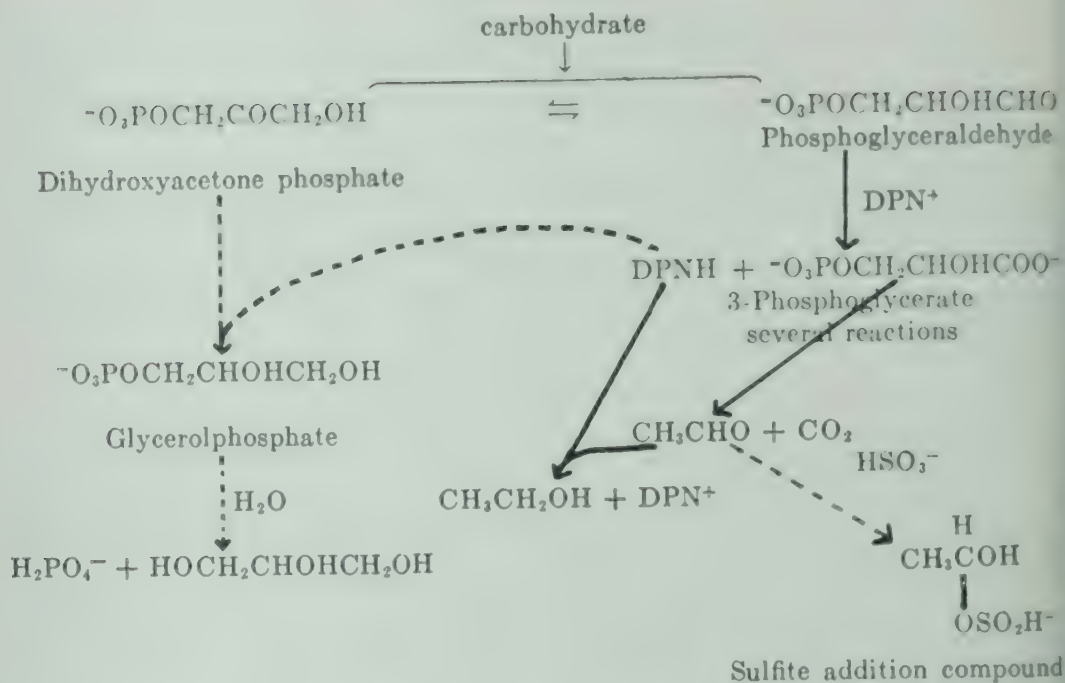
The now classic inhibition experiments involving the effect of malonate on animal tissues showed that inhibitors might be valuable tools in metabolic studies. When malonate is added to respiring liver preparations, the respiration is altered and succinate accumulates in the mixture. From these observations two points of information may be inferred. First, one suspects that succinate may play a role in respiration and secondly that the action of malonate is on the utilization of succinate.

The first hypothesis will be valid except when respiration is so altered as to yield a product not normally formed by the organism. This possibility may be illustrated in the case of yeast which ferments carbohydrate to acetaldehyde and glycerol in the presence of sulfite. The sulfite alters the normal metabolism by combining with the acetaldehyde and preventing the normal reduction of this compound to alcohol. This block now leaves the organism with reduced coenzyme I which becomes available for reduction of triosephosphate to glycerol. One mole of glycerol is obtained for each mole of the sulfite addition compound of acetaldehyde that is formed. The reactions described may be represented schematically as shown on next page. The dashed arrows represent the process occurring when sulfite is added. Glycerol evidently is formed as a result of the need for a hydrogen acceptor capable of oxidizing DPNH to DPN<sup>+</sup> which is required in the formation of 3-phosphoglycerate. The normal reduction of acetaldehyde to ethanol by the DPNH is prevented by removal of the aldehyde leaving dihydroxyacetone phosphate and phosphoglyceraldehyde as the next most effective oxidizing agents.

One would anticipate the reductions of the three carbonyl compounds and that at least a trace of glycerol would be formed in the absence of sulfite. This expectation actually is fulfilled since a small amount of glycerol is formed by yeast during the normal fermentation of carbohydrate.

Even though the sulfite does not act on glycerol itself, the inhibitor does cause accumulation of a substance normally present. In this way a com-



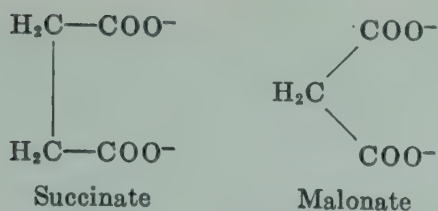


pound has been revealed that otherwise might be overlooked since it occurs in only small quantities in the natural fermentation. The trapped acetaldehyde; on the other hand, reveals the actual point of the inhibition and is, of course, a normal intermediate in the major pathway of fermentation.

The second inference drawn from the effect of malonate on respiring liver slices involved the site of action of the inhibitor. Except in situations like that of the glycerol from yeast one would anticipate that a compound accumulates because its direct utilization is blocked. Succinate ordinarily is oxidized to fumarate by means of succinic dehydrogenase, an enzyme transferring a molecule of hydrogen to acceptors like methylene blue or the cytochromes which probably act as the hydrogen acceptors in natural systems. If the components, represented by reaction (1) where A is a suitable hydrogen (or electron) acceptor, are brought together the process goes to an equilibrium state shifted far to the right:



If malonate is placed in the system before the succinic dehydrogenase is added, the reaction is blocked or inhibited. Such interference is ascribed to the inability of the enzyme to split malonate in spite of a strong tendency of malonate and the enzyme to combine. Apparently the succinate and malonate structures are sufficiently alike



to permit attachment of either one to succinic dehydrogenase. Yet from the structure it is clear that malonate cannot be dehydrogenated according to the general scheme of reaction (1). Therefore, the enzyme complex with malonate will not undergo reaction and by tying up the enzyme will actually prevent an attack on succinate.

According to modern theories the two compounds compete for the same enzyme with the establishment of some state of equilibrium. In the example under discussion malonate has nine times the affinity for the succinic dehydrogenase of animals that succinate has. Hence reaction (1) will be essentially stopped until succinate accumulates in sufficient quantity to permit successful competition for enzyme. If succinate and malonate are equal in quantity only ten per cent of the enzyme will combine with succinate, and the rate of reaction will be correspondingly low. In this way succinate is formed faster than it is oxidized and continues to accumulate at least until its concentration considerably exceeds the concentration of malonate.

On the other hand the succinic dehydrogenase of *Escherichia coli* has three times the affinity for succinate that it has for malonate. Therefore, malonate has little effect on reaction (1) in these bacteria unless it is present in high concentration. Unfortunately, if the concentration becomes too high this substance begins to affect other systems. Hence malonate is not as useful in studies of *Escherichia coli* and many other bacterial species as it has been in studies of animal tissues.

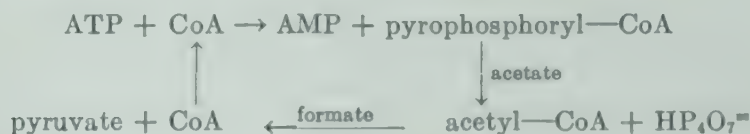
Inhibitors act in several ways. They may react with a substrate or an enzyme. In the former case the substrate is so modified as to prevent its normal reaction as illustrated by the action of sulfite on acetaldehyde in fermenting yeast. The acetaldehyde is precipitated and cannot be reduced. Malonate serves as an example of a compound which acts as an inhibitor by reaction with an enzyme to yield an inactive complex and so preventing normal enzymatic activity. Reactions of inhibitors with enzymes is a field of great importance and is discussed again in the chapter on chemical disinfection.

### *Isolation and Chemical Determination*

These two classes of methods may be conveniently discussed together because isolation ordinarily is achieved with the concurrent chemical determination of the factor in question. Furthermore, both are used widely

as portions of other procedures. For example, glycerol is found to accumulate in quantity during the inhibition of the fermentation of yeast simply by isolating and characterizing it or by the quantitative determination of glycerol in extracts. In other words, isolation and determination are valuable adjuncts to many kinds of metabolic studies.

Work done with extracts of *Escherichia coli* will serve to illustrate the application of typical procedures. It was reported that such extracts were able to convert acetate and formate to pyruvate. This can be shown readily by a quantitative analysis for acetate, formate, and pyruvate. The first two should disappear on incubating them with the cellular extract and concomitantly the pyruvate should increase over the value at zero time. Once these changes are established the mechanism of the process may be investigated, still using chemical analyses to follow the progressive changes. It has been shown in this instance by eliminating various substances from the system that other factors are required, namely, inorganic phosphate, coenzyme A, cocarboxylase, ATP, and more than one enzyme. A hypothetical process may be written:



In this scheme ATP and AMP represent adenosine triphosphate and adenosine monophosphate respectively. CoA is coenzyme A to which a pyrophosphate group is attached by the top reaction. This product in turn reacts with acetate to yield acetylated coenzyme A and becomes the so-called "active acetate". Formate then combines with the acetyl group to form pyruvate, and the coenzyme A is ready for reuse. The roles played by cocarboxylase and inorganic phosphate are not yet known, hence the actual process is more complex than shown. The enzymes that catalyze the various steps are not completely characterized.

The literature cites a number of cases, mostly among the higher animals and plants, from which compounds have been isolated without any knowledge of their biological function. During subsequent investigations these substances have been shown to possess important biological activities. One large group of compounds in this stage of study includes the alkaloids. These compounds have been isolated in large numbers from plants and the structures of many have been established. However, the role of the compounds in nature is still not known.

More often a phenomenon has been observed, and an agent participating in the phenomenon has been isolated and studied. Sometimes entirely unknown materials are obtained while on other occasions materials of



known chemical composition and structure are isolated and shown to have previously unknown functions.

The discovery of vitamin B<sub>12</sub> may be used to illustrate the isolation of new substances. The entire course of investigation began when liver extracts were found to contain a factor that was effective in the treatment of anemia in humans. Efforts made to isolate this factor led ultimately to its separation in large quantities from the culture media of molds. When purified it was readily shown to be a hitherto unknown compound, so studies of its structure were begun. At the same time the biological action of the vitamin was investigated, and it was found to be important in the metabolism of all species studied. For many species B<sub>12</sub> must be added to the medium, including bacterial species like *Micrococcus lysodeikticus* as well as for humans for whom the requirement was originally discovered.

*p*-Aminobenzoic acid has been studied chemically for a century but was not known to be a growth factor for bacteria until 1940. The compound has now been shown, starting first with *Clostridium acetobutylicum*, to be required by many bacterial species. Still other species require it but synthesize the necessary quantities. From these latter species the factor may be isolated in the manner first applied to its isolation from yeast. This compound, long used for synthetic purposes in the organic chemistry laboratory, serves to illustrate how new biological functions may be discovered by new isolations of otherwise well known substances.

In recent years the classical methods of direct chemical determination and isolation have been greatly extended by the development of new techniques. Two of the most widely used are the methods of *chromatography* and *isotopic tracing*. Since these procedures have been so extensively developed and applied and have led to important advances in our knowledge of metabolism, they will be discussed in the next section.

### *Chromatographic and Tracer Methods*

Chromatography is an adsorption process carried out in a differential countercurrent manner. In such a process the material to be analyzed is placed on an adsorbent. Theoretically the adsorbent can be any substance with which the test material combines reversibly. The system is then washed with a solvent (called the *developer*) in such a way that the solvent sweeps across the adsorbed material onto fresh adsorbent. In this way a portion of the adsorbed material is dissolved in the moving solvent, transported through the zone of adsorption, and readsorbed beyond the edge of the zone. Thus adsorbed material is moved along the adsorbent, and any different substances present, that differ in the tightness with which they are adsorbed, move at different rates and are eventually separated.

There are four kinds of useful observations that can be made with

chromatography. First, this method is a good criterion of purity and is used to verify the homogeneity of materials. For this purpose the substance in question is moved through an adsorbent by means of a developer and a second discrete zone of adsorbed material sought. If none is found a second adsorbent-solvent system is used inasmuch as two different compounds will sometimes migrate together in one system but usually will separate in another. This method is an excellent test of the purity of molecules up to several hundred in molecular weight. Mixtures of large compounds such as proteins have been studied but little and do not appear to be as readily resolved as the smaller substances. Furthermore, proteins are often subject to permanent alteration by adsorption on many materials, and developers are largely limited to aqueous solutions in which the proteins are soluble and stable.

Since materials may be separated by chromatography, the process can be used for the isolation of substances. For this purpose development may be continued until the given substance is washed off a column of adsorbent by the moving solvent after which the appropriate fraction is identified. On the other hand the chromatographic column may be developed, the zones located and separated mechanically, and adsorbed materials extracted from the individual zones.

The quantitative analysis of a mixture may be undertaken by chromatographic methods. First the components are separated, as outlined above. Then they are either determined in place on the chromatogram by means of appropriate analytical procedures such as color reactions, or the components are separately isolated and determined by chemical methods, refractive index, weight, and so on. Methods have been described for estimating the quantity of material by means of the size of the zone of adsorbed material produced under standard conditions. These procedures are usually not accurate and are difficult to control suitably making them less useful than a method of actual isolation and determination.

Finally, chromatography is quite useful for the identification of compounds. Under a given set of conditions the distance a substance moves along an adsorbent is a characteristic property. Because it is frequently difficult to reproduce the conditions of an experiment exactly, standard substances are usually included in the system for comparative purposes.

The experimental variations so far reported in the literature for the use of chromatography have now become exceedingly numerous and cannot be summarized here. The general features are discussed and specific details referred to in the book by Cassidy entitled *Adsorption and Chromatography*. In all cases the adsorbed material is held at an interface between two phases. Since the developer is a pure liquid or a solution, one of the phases is liquid. The other phase either is a solid or is composed of a solid on which



a liquid is immobilized. This latter situation is commonly called *partition chromatography* and appears to depend upon a distribution of solutes between the immobilized liquid and the moving liquid phase.

An example of the use of partition chromatography is shown in Figure 80. Here the great separating power of the method is clearly revealed. The method used permits the separation, isolation, recovery, identification, and characterization of fifteen fractions, all but three of which are pure materials. The three mixtures may be subsequently separated by re-chromatographing them individually with another adsorbent-developer

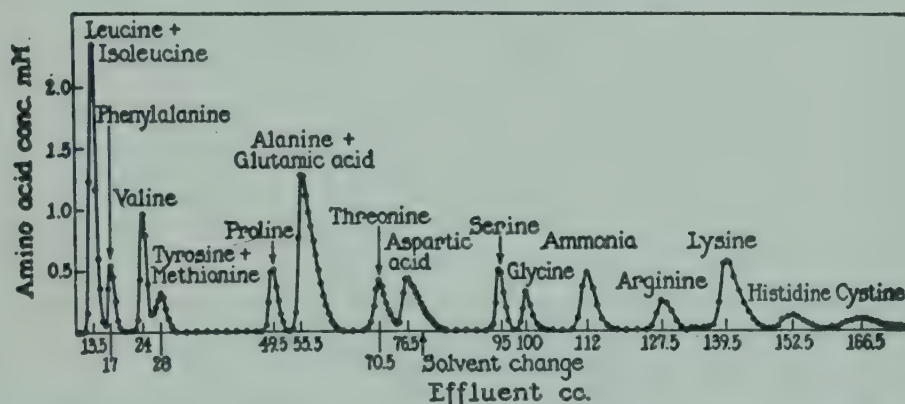


FIG. 80. Chromatographic fractionation of a hydrolysate of bovine serum albumin. The solvents were first a 1:2:1 mixture of *n*-butanol, *n*-propanol, and 0.1 M HCl; changed as indicated by the arrow to a 2:1 mixture of *n*-propanol and 0.5 M HCl. The column itself was potato starch. Each point represents a fraction of the effluent from the column as the solvent passed through, and all fractions were analyzed for the presence of amino acids.

(From Stein and Moore, 1949)

system. This example illustrates some of the flexibility introduced into chromatographic methods when more than one developer is employed.

For qualitative studies an important type of partition chromatography has been developed and is called *paper chromatography*. In modifications of this procedure a portion of the sample is placed at a spot on strips or sheets of filter paper. The paper then is brought into contact at one edge with the developer which moves through the paper by capillarity and acts upon the sample as it passes. Quite commonly one of the liquids in the developer is held on the surface of the cellulose and becomes part of the stationary phase. In such instances the process may be described best as a *counter-current liquid-liquid distribution*.

When sheets of paper are used the solvent moves in a front across them and resolves a small sample into spots located along a line in the direction of solvent motion. If the sheet is dried and rechromatographed in a new



solvent at right angles to the original motion, further resolution may be achieved. Thus, all of the spots first obtained may be readily tested for purity, and a large number of zones may be spread from a complex mixture. This process is called *two dimensional paper chromatography* and is widely used.

Although paper chromatography cannot be used with large quantities of material, it is ideal for the detection of compounds for which there are sensitive tests. The methods of this kind are useful in metabolic investigations for revealing the presence of intermediates. In addition the order of appearance or disappearance of metabolites may be determined by chromatographing extracts in which the reaction has been interrupted at varying times. Identification of the compounds separated and a correlation with time will establish the order of appearance of the substances.

Figure 81 illustrates the use of two dimensional paper chromatography. The zones were located in this example by application of ninhydrin which forms colored compounds with amino acids. In this study diaminopimelic acid was identified for the first time from a natural source. Paper chromatography was then used to follow the separation of the amino acid on columns of alumina adsorbent from which the amino acid was obtained in pure form and could be definitely characterized.

Another type of fractionation process that involves a liquid-liquid partition is known as *countercurrent distribution*. Here, however, solid is not used and the solute is distributed between two immiscible liquids. The two liquids are mixed until the solute reaches an equilibrium distribution whereupon the phases are separated. Each phase is then brought into contact with a fresh portion of liquid of the opposite type and the mixing and separation are repeated. After many such stages a mixture of solutes is resolved into separate components if these components differ in their equilibrium distribution between the two immiscible solvents employed.

Devices of various kinds have been described for systematically separating the immiscible layers and moving them progressively until each upper solution has been brought successively to equilibrium with each bottom solution. In this way a given solute is distributed in a characteristic pattern among the successively employed fractions of the solvents, and a mixture of substances whose solubility properties differ will be resolved into the individual compounds.

In general, countercurrent distribution will serve the same purposes as partition chromatography. However, it does have the disadvantages of requiring relatively expensive apparatus and of showing less rapid resolution than two-dimensional paper chromatography. On the other hand, fairly large quantities of materials may be employed, and the materials separated are recovered by merely removing the solvent.

The well known methods involving radioactive indicators are now employed extensively in studies of metabolism. The various instruments used to detect nuclear reactions allow one to follow the assimilation or excretion of unstable isotopes of most elements. The isotopes may be incorporated into ions or into compounds of any kind. By determining the fate of the tracer atoms one may study the transfer and storage of the intact material, the nature of products formed from the tagged or labeled sub-

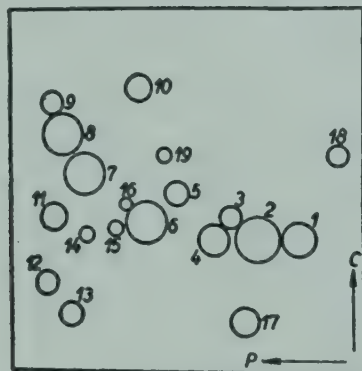


FIG. 81. A tracing of a two-dimensional paper chromatogram of the acid hydrolysate of the acetone-alcohol insoluble residue of *Corynebacterium diphtheriae*. Motion in the direction indicated by the arrow marked P was produced by phenol saturated with water, and the experiment was conducted in an atmosphere of ammonia. Arrow C indicates movement produced by a mixture of collidine and lutidines saturated with water. The spots were identified as follows: 1. aspartic acid, 2. glutamic acid, 3. serine, 4. glycine, 5. threonine, 6. alanine, 7. valine, 8. leucines and methionine, 9. phenylalanine, 10. tyrosine, 11. proline, 12. arginine, 13. lysine, 14. methionine sulfoxide, 15. histidine, 16. hydroxyproline, 17. subsequently identified as  $\alpha, \epsilon$ -diaminopimelic acid, 18. cysteic acid, and 19. unidentified. Thus, the protein of this species contains 19 amino acids (including leucine and isoleucine) plus one unidentified substance and probably tryptophane which is destroyed by the acid hydrolysis. (From Work, 1949)

strates, or the intermediate compounds and the synthetic or degradative processes taking place when the substrate material is used biologically.

Often radioactivity may be measured accurately even though a number of different non-radioactive compounds are present. Thus difficult separations are avoided. Since it is rather easy to measure a low level of radioactivity, a small amount of a compound containing an unstable isotope may be determined. In this connection the method of *isotopic dilution* is a valuable adjunct. This procedure allows the determination of a substance in a mixture when a known quantity of the substance in question is added to the mixture, providing the added sample is chemically pure, radioactive, and has a known specific activity. *Specific activity* refers to the fraction of a substance containing the radioactive atom. It is usually



expressed simply as counts per minute per unit weight which is proportional to the number of nuclear disintegrations per minute per unit weight. After the added compound with the radioactive isotope has become mixed to equilibrium with that originally present, a sample is isolated, purified, and the specific activity determined. From these data the original quantity of material in the sample may be calculated since the reduction in the specific activity of the labeled sample added to the mixture depends only upon the ratio of the quantity of substance originally present to the weight of labeled compound added. The isolation required need not be quantitative but must yield a pure product.

Labeled compounds may be added to any reacting system and the various products isolated and tested for radioactivity. In this way the products deriving from the test substance may be related to it. Since metabolic processes are sometimes very complex, the substrate compound may be labeled in more than one way with a particular isotope or with isotopes of more than one element. This procedure allows one to relate products of metabolism to particular parts of the original molecule and aids in the search for the specific reactions involved.

Tracers are convenient in rate studies designed to reveal the order in which the individual reactions in a series occur. There are no differences in principle when isotopes are used as compared to the methods with stable compounds discussed earlier.

Whereas the foregoing comments have been based on radioactive atoms as indicators, the same principles and methods may be applied with stable isotopes whose atomic weights differ from the natural average weights of atoms. Indeed tracer studies with nitrogen as the label depend upon such a stable isotope, and most of the work with hydrogen labels has been done with the stable deuterium, since radioactive isotopes are not readily available in these two cases. The stable isotopes may not usually be determined in such small amounts as the radioactive atoms, and in general the measurements are slower and require more elaborate instruments. On the other hand there is no danger of radiation injury to laboratory personnel, of changes induced in biological systems sensitive to radiation, or of a decrease with time in the sensitivity of methods of analysis for the stable isotope.

It should be emphasized that isotopes of abnormal weight differ slightly in chemical properties from the natural mixture of isotopes of a given element. This difference is greatest with the light elements and may conceivably affect reactions. It will be recalled that some enzymes which normally transfer hydrogen atoms at given rates can transfer deuterium but at only lower rates. When interpreting experiments in which rates may play a part, this nonidentity of isotopes should be kept in mind.

Some investigators have combined radioactive tracer work with paper



chromatography. By this means zones on two-dimensional chromatograms may be readily located by the presence of radioactivity thus avoiding the need for analytical chemical tests which may not be available for all the

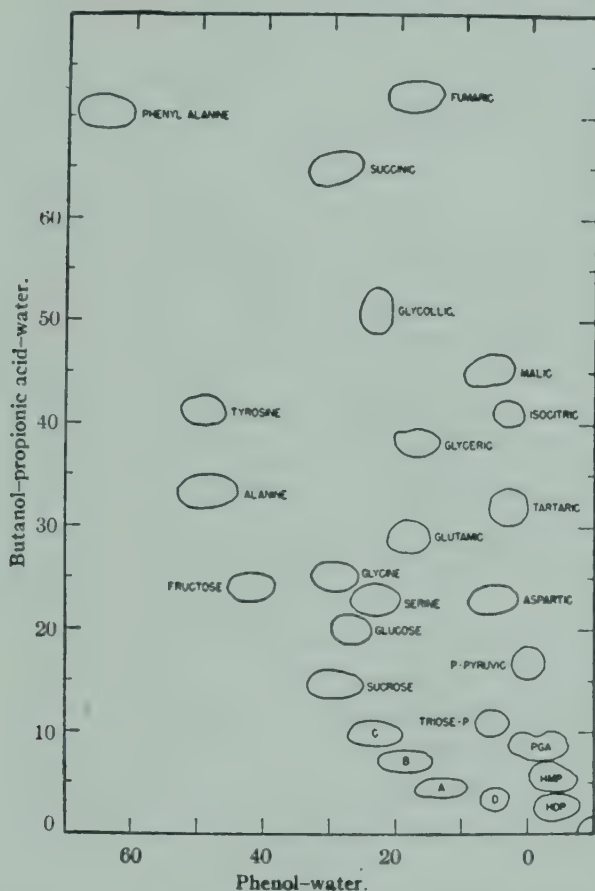


FIG. 82. Soluble products of photosynthesis revealed by radioautography after two-dimensional chromatography in the indicated solvents. PGA, HMP, and HDP refer respectively to phosphoglyceric acids, hexose monophosphates, and hexose diphosphates. A, B, and C are unidentified compounds containing glucose, and D is an unidentified compound containing glucose and glucose phosphate. The radiogram is prepared from the chromatogram by placing the latter against a sheet of sensitive film until the radioactive zones produce corresponding exposures of the photographic emulsion. These zones appear as darkened areas when the film is developed.

(From Benson et al., 1950)

compounds present. Figure 82 is a tracing revealing the relative positions of typical biological compounds in a two-dimensional chromatogram. These substances were extracted with ethanol-water from dried algae (*Scenedesmus*) which had previously carried out photosynthesis in the presence of carbon dioxide labeled with radioactive carbon. This figure shows the fate

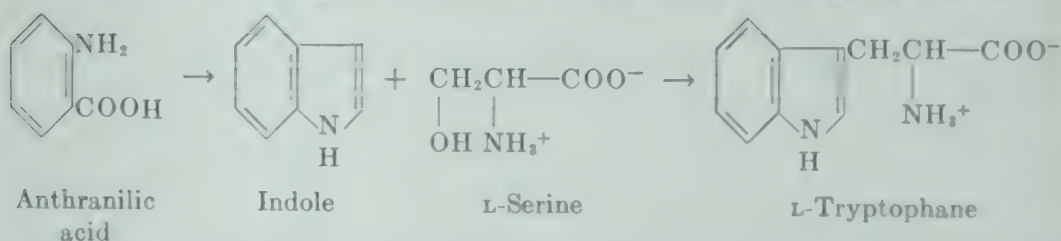
of carbon incorporated into the soluble components of the cell. It will be recalled that rates may be critical factors for the synthesis of certain compounds which either have not yet become radioactive or have lost all of the radioactivity by rapid utilization of these substances after photosynthesis ceased. Hence one cannot assume without making extensive rate studies that the substances observed in Fig. 82 are the only compounds involved in photosynthesis.

### Mutation

The determination of reactions in which metabolites participate and of metabolic pathways for both synthesis and breakdown of substances may be studied using mutant strains of bacterial species varying in their growth factor requirements. While mutants may be recovered from nature the more usual expedient is to expose bacterial cultures to mutagenic agents in order to increase the chances for the isolation of desired mutant types. Ultraviolet and x-rays have been employed most frequently, and at high dosages so that while most of the irradiated bacteria are killed the proportion of mutants among survivors is high.

Study of the nutritional needs of mutants and of the capacity of postulated intermediate metabolites to replace required nutrients provides the basic data for the intellectual construction of metabolic pathways. The basic premise of the method of construction is the assumption that intermediates located at reaction stages beyond the point of the metabolic block represented by the deficiency in synthetic capacity of the mutant can successfully replace the requirement for the specific nutrient. On the other hand, intermediates participating in reactions preceding the blocked stage at which the nutrient is utilized cannot replace the need for the nutrient. The principle is illustrated by the experience obtained with mutants of *Escherichia coli*, *Salmonella typhosum*, and the mold species *Neurospora crassa* in the study of the problem of the biological synthesis of tryptophane.

A tentative and logical scheme for the synthesis of tryptophane is



*Escherichia coli* of the wild type can synthesize tryptophane in a synthetic medium containing an ammonium salt and glucose as sole sources of nitrogen and carbon respectively. Mutants requiring tryptophane can be iso-

lated. Among these are two kinds: 1) A mutant which can utilize either anthranilic acid or indole in place of tryptophane; 2) a type which can utilize indole in place of tryptophane but not anthranilic acid. When tryptophane is supplied to the latter type of mutant and growth results, it is found that anthranilic acid accumulates in the medium. This would be expected if the requirement for tryptophane resulted from a block at the reaction involving the conversion of anthranilic acid to indole. The general principle holds that accumulation of a precursor takes place when there exists a deficiency in the utilization of the precursor. Mutants were not isolated which could utilize anthranilic acid and not indole and tryptophane. In the case of a *Neurospora* mutant it was found that in a medium containing indole but free of serine, tryptophane was not synthesized but was synthesized when both serine and indole were present. All these data plus other experiences have established the existence of the above postulated mechanism for the synthesis of tryptophane.

The basic genetic concept originally underlying the use of mutants for the study of intermediate metabolism is the *one enzyme-one gene* theory. The notion is that a gene acts by controlling the synthesis of one enzyme. Thus a single gene mutation will result in the loss of only one enzyme and consequently will lead to a metabolic block at the single step at which the enzyme participates in a metabolic chain of reactions.

The one enzyme-one gene theory cannot be considered to be firmly established and is still subject to further development and experimental verification. In the case of *Escherichia coli* a mutation possibly pleiotropic has been described, a single gene mutation affecting the production of several enzymes including lactase. In addition some lactase negative mutants have been shown actually to produce lactase under certain conditions when the bacilli were exposed to a variety of growth temperatures and substrates. Furthermore, there is evidence to suggest that genetic metabolic blocks are relative and do not necessarily involve an absolute loss of an enzyme. In the case of both *Neurospora* and bacteria, mutants originally thought to lack the ability to synthesize a particular growth factor have been shown to synthesize the required factor at a low rate. *Leakage* has been the term applied to this phenomenon.

Observations of the above character have cast doubt on the validity of the original *one enzyme-one gene* concept. In an effort to rescue the hypothesis it has been suggested that a gene acts primarily on the formation of a single enzyme but that other genes may affect this synthesis secondarily. When it is recalled that all intermediate metabolism including protein synthesis is an intermeshing network of numerous series of reactions such a possibility seems reasonable. The difficult problem is the invention of



criteria for distinguishing which gene among a number of influential genes has the primary effect on the synthesis of an enzyme.

In spite of the theoretical difficulties with the one enzyme-one gene theory the value of mutants for the study of problems of intermediary metabolism has been firmly established. The conceptional difficulties with the theory merely emphasize a situation that is true for all methods of investigation of intermediary metabolism, namely, the desirability of checking the results obtained by one method against data derived from studies with other possible methods.

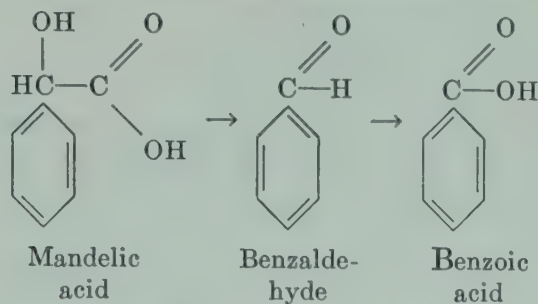
### *Simultaneous Adaptation*

In principle certain metabolic pathways may be traced by testing for the presence of adaptive enzymes acting on postulated intermediates in bacteria which have been grown on various media each containing only one of the intermediates. The first requirement of course is the availability of a bacterial culture having an adaptive enzyme system for the particular metabolic activity under investigation. The great variety of bacterial species and the diversity of dissimilatory processes existent among the eubacteria favor the prospects for the isolation of strains with desired adaptive enzyme systems. In this regard the genus *Pseudomonas* has proven most prolific.

The theory of simultaneous adaptation rests on the premise that in the dissimilation of a substrate not only will the first or primary step be controlled by an adaptive enzyme but so will the immediately following reactions. Inasmuch as an intermediate metabolite may not be peculiar to a particular metabolic chain of reactions but may originate by a number of means, the adaptive nature of the enzymatic reaction involving each of the intermediates must be established for every system studied. The synthesis of adaptive enzymes by a culture grown in the presence of a particular substrate will be *limited* to those enzymes acting on the substrate and those intermediate metabolites following in the reaction chain. The organism will generally not synthesize enzymes whose sites of action are located at reaction stages coming before the place at which the substrate on which the culture was grown is located. Thus in the metabolic series  $A \rightarrow B \rightarrow C \rightarrow D$  a culture grown on *A* possesses enzymes capable of acting on *B*, *C* and *D* as well as *A*. But a culture grown on *D* usually will not synthesize enzymes acting on *A*, *B* and *C* since these substrates are not present either exogenously or endogenously and therefore the requirement for adaptive enzyme formation, the presence of specific substrate, is not met.

*Pseudomonas fluorescens* oxidizes mandelic acid to benzoic acid by an

adaptive enzyme system in the following manner:



Resting cell suspensions of *Pseudomonas fluorescens* grown originally on mandelic acid oxidize both benzaldehyde and benzoic acid without a lag period being observed when tested manometrically. This indicates the presence of enzymes acting on these substrates in the bacteria when grown on mandelic acid. On the other hand, a resting cell suspension from a culture grown on benzoic acid is able to begin oxidizing mandelic acid and benzaldehyde only after the suspension has been in contact with the substrates for a period of time. This means that the organisms, though potentially capable of forming adaptive enzymes for the oxidation of mandelic acid and benzaldehyde, do not do so when they are grown on benzoic acid. Bacilli grown on benzaldehyde attack benzoic acid on immediate contact but will only act on mandelic acid after a lag period. Therefore, the observations are taken as an indication that the oxidation of mandelic acid to benzoic acid proceeds through benzaldehyde as an intermediate stage.

Experience with the technique of simultaneous adaptation has indicated that negative findings cannot be taken at face value. The inability of bacteria to act upon an exogenous source of a postulated intermediate is not always due to the absence of a necessary enzyme. Not infrequently it actually is due to the fact that the organisms are impermeable to the intermediate.

The existence of equilibria in enzymatic systems makes it difficult to categorically fit intermediates into their proper sequence in a metabolic chain of reactions based solely on the data of simultaneous adaptation. Growth of bacteria on one of two compounds in equilibrium may result in the formation of the adaptive enzymes acting upon both compounds. This is apparently the case with *Pseudomonas aeruginosa* adaptively oxidizing glucose. Resting cell suspensions of these bacilli harvested from media containing either glucose or gluconic acid are equally capable of immediately oxidizing both substances at maximum rates. The same is true for the pair, pyruvic acid-acetic acid. This interrelationship is presumably due to the existence of equilibria, glucose  $\rightleftharpoons$  gluconic acid, and pyruvic acid  $\rightleftharpoons$  acetic



acid, which yield appreciable quantities of the missing substance when only one is included in the growth medium.

#### GENERAL PROPERTIES OF COENZYMES

In Chapter 9 enzymes were dealt with in a general way, and brief mention was made of coenzymes. Before proceeding further into a discussion of intermediary metabolism it is desirable to once again consider the nature of coenzymes.

The materials classified as coenzymes are quite diverse chemically, so that the actions in which they participate may be equally varied. It must be admitted that changes in the coenzymes themselves during catalytic activity can be shown in some but not in all cases. Indeed it is difficult to conceive of the nature of any change in such an essential coenzyme (or cofactor) as magnesium which is active in a number of enzyme systems.

On the other hand, there are coenzymes which undergo oxidation and reduction during the normal course of events. In such cases the coenzyme always performs the same general function regardless of the particular substrate and apoenzyme with which it is associated. Thus the coenzymes do have a functional specificity, being limited in their activity to certain types of chemical processes even when they act in different systems with different apoenzymes.

Coenzymes by their presence in an enzyme system may act to increase rates of reaction. Sometimes their roles may be defined more precisely as in cases where they are known to be the means whereby a specific substrate is attached to an enzyme during the reaction process. Still other coenzymes may function as regenerating substances as in oxidation-reduction and phosphorylation-dephosphorylation reactions. In these cases the coenzyme may be oxidized or phosphorylated at one stage in a sequence of reactions and reduced or dephosphorylated at another step in the same reaction series.

Since in any one enzyme system several roles may exist for the coenzymes, more than one coenzyme actually may participate in a single enzyme system. The hexokinase reaction which requires both magnesium and ATP may be cited as one example of such a situation.

Enzymes, of course, are quite specific with regard to the substrates effectively utilized and so far as is known are also specific to a great extent toward any associated coenzymes required for their activity. A given enzyme or apoenzyme seems to have no ability whatever to act with more than one of the organic coenzymes. Yet they may be active with one of several metallic cations as cofactors.

The other sort of situation, namely, that a given coenzyme occurs with more than one enzyme, is apparently rather commonplace. One may con-



clude then from these remarks that coenzymes confer a certain amount of general specificity on the coenzyme-apoenzyme complex for the substrate but that it is the apoenzyme which is responsible for specificity in detail.

Table 46 lists a number of enzyme-coenzyme systems, revealing that several different enzymes may operate with a particular coenzyme. The names of the enzymes reveal the specificity of the system to some extent; actually only a single known compound can serve as a substrate in particular cases. It should be understood that the table does not pretend to include all of the known coenzymes nor all of the enzymes with which any one coenzyme may be associated.

The first five coenzymes listed in Table 46 belong to the class of compounds called nucleotides and are listed in the table in the abbreviated form that has had universal acceptance. Nucleotides consist of at least three separable portions united by condensation reactions which split out water. A heterocyclic base, a sugar, and phosphate are formed when an ordinary nucleotide like ATP is hydrolyzed. Some of the compounds ( $\text{DPN}^+$  and  $\text{TPN}^+$  for example) are dinucleotides formed by combining two simple (mono) nucleotides. The sugar is always D-ribose or the alcohol D-ribitol formed by reduction of this carbohydrate. A single phosphate is found in the true mononucleotides but certain of the coenzymes contain two or three phosphate residues. ATP is one of these as indicated by the name from which the abbreviation is taken, adenosine triphosphate. The other abbreviations are paired with the more complete names as follows: ADP, adenosine diphosphate;  $\text{DPN}^+$ , diphosphopyridine nucleotide;  $\text{TPN}^+$ , triphosphopyridine nucleotide; CoA, coenzyme A. The last is a compound of adenosine monophosphate and pantothenic acid. Rather than discuss the structure and properties of these and the other coenzymes in detail in this section, they will be treated separately in connection with various important enzyme systems in which they occur.

#### CARBOHYDRATE METABOLISM

More effort has been expended in the study of the metabolism of carbohydrates than any other type of substrate. As a result our knowledge of the process of carbohydrate utilization has become quite extensive though certainly not yet complete. In assembling the information on metabolism into an integrated scheme it has been helpful to assume that different species carry out the same reactions. Indeed, this assumption is justified to some extent by repeated observations that such is the case. Hence diagrammatic representations of metabolic processes have always been based upon data from a variety of species depending upon the convenience or economics of the moment.

Inasmuch as a close parallel is observed between many species, there has

been some tendency to assume that all organisms metabolize a given compound in the same way. This hypothesis seems to be an oversimplification, especially for the bacteria and has occasionally obscured the actual situation. In view of the increasing evidence of species variations it behooves the

TABLE 46  
*Some coenzymes functioning in various enzyme systems*

COENZYME	ENZYME
ATP.....	Hexokinase
ATP.....	Phosphohexokinase
ATP.....	Condensing enzyme
ADP.....	Phosphoglyceric phosphokinase
ADP.....	Pyruvic phosphokinase
DPN <sup>+</sup> .....	Malic dehydrogenase
DPN <sup>+</sup> .....	L-Glutamic dehydrogenase
DPN <sup>+</sup> .....	Lactic dehydrogenase
TPN <sup>+</sup> .....	iso-Citric dehydrogenase
TPN <sup>+</sup> .....	Glucose-6-phosphate dehydrogenase
CoA.....	Condensing enzyme
Mn <sup>++</sup> .....	Oxalosuccinic dehydrogenase
Mg <sup>++</sup> .....	Enolase
Mg <sup>++</sup> .....	Hexokinase
Mg <sup>++</sup> .....	Phosphorylase
HPO <sub>4</sub> <sup>-</sup> .....	Phosphorylase
Pyridoxal phosphate.....	L-Lysine decarboxylase
Pyridoxal phosphate.....	Transaminase
Glucose-1,6-diphosphate.....	Phosphoglucotransferase

student to bear in mind that the current ideas of metabolic mechanisms must be merely tentative until the details are known for the individual species. Among closely related species the differences in metabolism are probably slight, as seems to be the case among the mammals, for example. The more distant the evolutionary kinship the more cautious must one be in extrapolating from species to species. Yet throughout living forms there are common elements. The carbohydrate metabolism of the muscle tissue of birds and mammals seems to be alike although differences do exist

between other metabolic processes such as nitrogen excretion of these two classes. The green plants carry out many of the reactions and synthesize many of the intermediates that are important to other forms, both plant and animal. Under certain environmental conditions yeast and muscle utilize carbohydrate in a similar way, but manipulation of the environment reveals important differences in the operation of these different materials.

Metabolism will be discussed with reactions shown either as they are known or are thought to occur in bacteria. It must be emphasized that the overall representation of metabolic pathways and cycles presented has not been established for any one bacterial species. Nor have all the individual steps been demonstrated in any single bacterial species. Rather the reactions outlined are logical combinations and extensions of data from a variety of organisms. They are brought together in an effort to integrate thinking and to summarize rapidly accumulating knowledge. It should be emphasized that the literature has now become so extensive that only a general survey is possible here with all the omissions inherent in a survey. Furthermore, the rapid progress being made in the understanding of intermediate metabolism will fill gaps of which we are not now aware. One should refer, therefore, to the various literature reviews appearing annually in order to keep the overall picture up-to-date.

In the ensuing discussion carbohydrate metabolism has been divided for the sake of convenience into aerobic and anaerobic mechanisms which, of course, are related. Of these two divisions, anaerobic metabolism has received the most attention probably because the processes carried out in the absence of oxygen are simpler than in aerobic metabolism. In addition, it currently appears that there may be greater variations in the mechanisms of species metabolizing aerobically than anaerobically. At any rate, knowledge of the aerobic metabolism of bacteria is less detailed.

### *Anaerobic Mechanisms*

Muscle may function at least temporarily under anaerobic conditions and succeeds in converting carbohydrate into lactate with a gain in energy available for work. This reaction process is called *glycolysis*, and this term is applied to the related anaerobic activities of microbes, also variously referred to as fermentations, or the anaerobic metabolism or dissimilation of carbohydrates. Among bacteria there are strictly anaerobic species whose multiplication is prevented by oxygen, and other species which require an added hydrogen acceptor such as oxygen for multiplication. However, the majority are facultative organisms capable of both the fermentation and the aerobic utilization of carbohydrate. In at least some facultative species the aerobic dissimilation appears to proceed from the stage where the



reactions associated with the fermentation of substrates end. The considerations of energy changes and transfers accompanying carbohydrate metabolism will be summarized in the discussion of aerobic mechanisms to permit a ready comparison of the anaerobic and aerobic processes, and because the aerobic processes provide much more energy per unit of dissimilated carbohydrate.

Throughout the discussion an attempt has been made to designate reaction products with names indicating the state of the ionizable groups of these products. For example, in the pH range within which the reactions are occurring biologically, pyruvic acid is largely in the form of the pyruvate ion. In compounds with two or more ionized groups it is not convenient to indicate the ionic state of all groups. Hence, such names as 3-phosphoglycerate have become conventional. In this case it is meant to imply that both the phosphate and carboxyl groups are ionized though strictly speaking the term glycerate only indicates the ionization of the carboxyl radical.

Figure 83 summarizes a part of the postulated mechanism of fermentation as brought about by bacteria. The only bacterial species for which the evidence of the above scheme is at all complete is *Escherichia coli*. Much study has been made of the metabolism of this organism and the figure indicates a portion of the results. The reactions shown are known to occur in this species with the exception of the formation of glucose-1-phosphate and fructose-1,6-diphosphate, and for these there is indirect evidence. The results represented have been obtained by use of the methods previously discussed, several of which usually have been applied to each step.

A number of the enzymes involved are known by more than one name, and some of these appear below. At the same time additional pertinent information is presented for several of the reactions starting with the carbohydrate and proceeding through the pathway. Because of spatial limitations not all of the general information nor all of the known reactions can be grouped into a single diagram. Therefore supplementary figures and a table covering the reactions of various carbohydrate substrates and the reactions of pyruvate have been employed to complete the general scheme of carbohydrate fermentation.

Phosphorylases are widely distributed enzymes, occurring in plants, animals, and bacteria. Little is known of the actual role played by these enzymes in bacterial fermentation, but it has been suggested with some supporting data that a phosphorylase acts on the cellular polysaccharide of *Escherichia coli*. As written in Figure 83 the process is represented as involving inorganic phosphate, magnesium ions, and adenylic acid (AMP). The participation of the last two factors is not clearly established but

rather is a logical inference from the accepted situation for muscle and yeast. The polysaccharide is assumed to contain glucose units since glucose-1-phosphate is formed from it by crude bacterial enzyme preparations. However, fructose-6-phosphate is also formed owing to the presence of the necessary enzymes in the impure preparations studied. In this situation

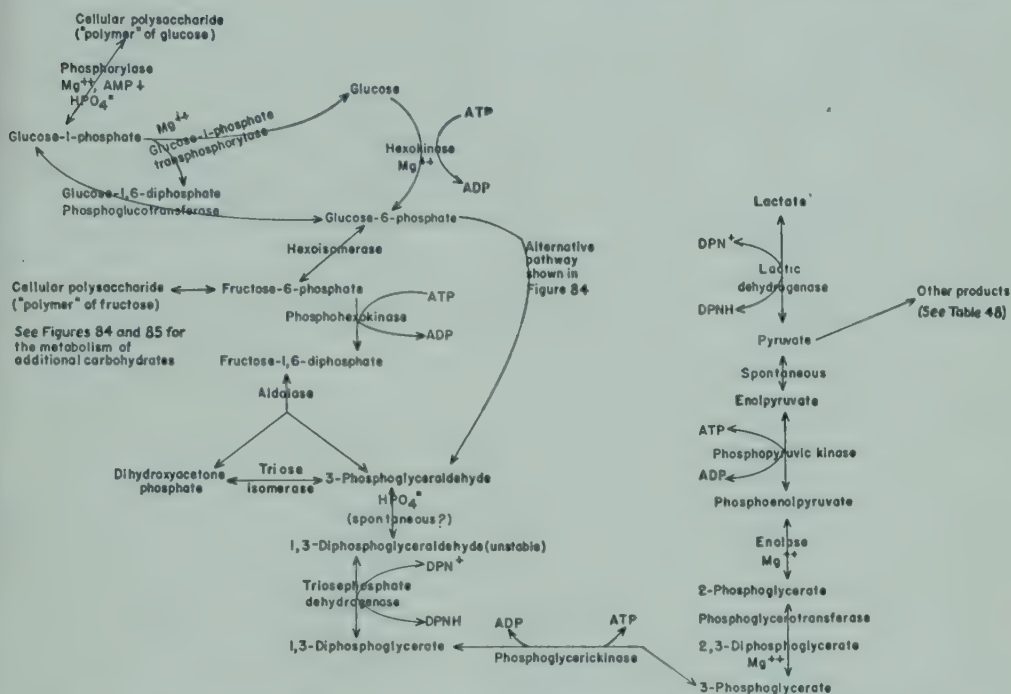
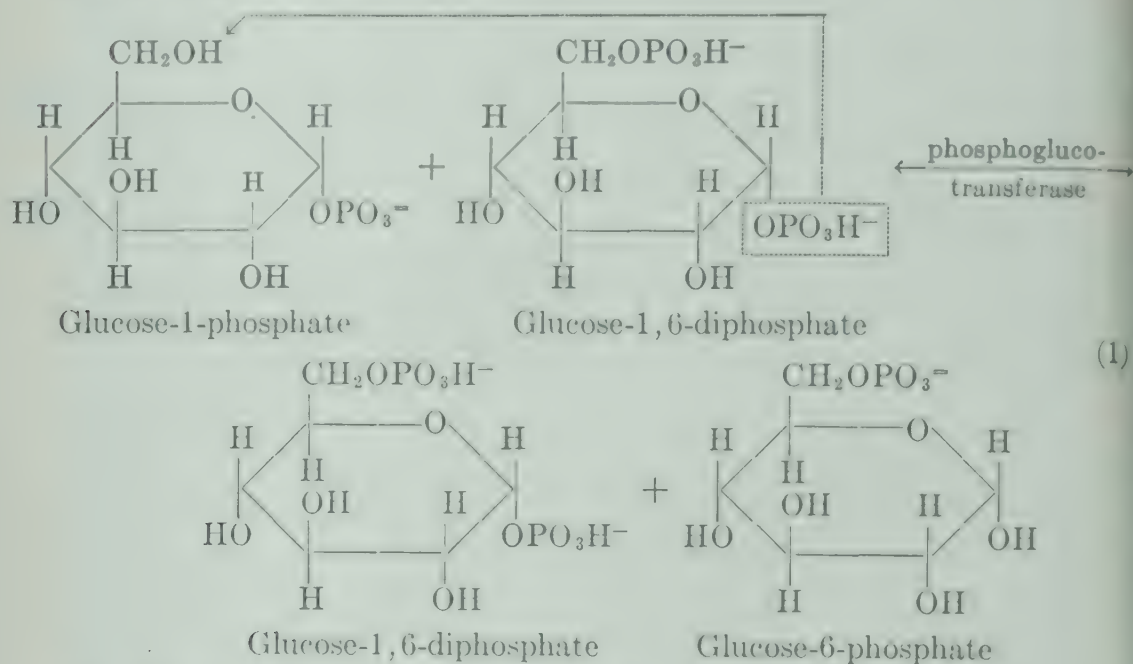


FIG. 83. A summary of the anaerobic metabolism of glucose and intracellular polysaccharide. Of the various end products produced from pyruvate only one is shown in this figure. The use of arrows pointed on both ends indicates reactions known to be reversible while a single point indicates the direction of a reaction not known to be reversible. Compound arrows show the participation of important cofactors and the changes these substances undergo. Additional cofactors that do not react in such obvious ways are listed with the enzymes with which they act.

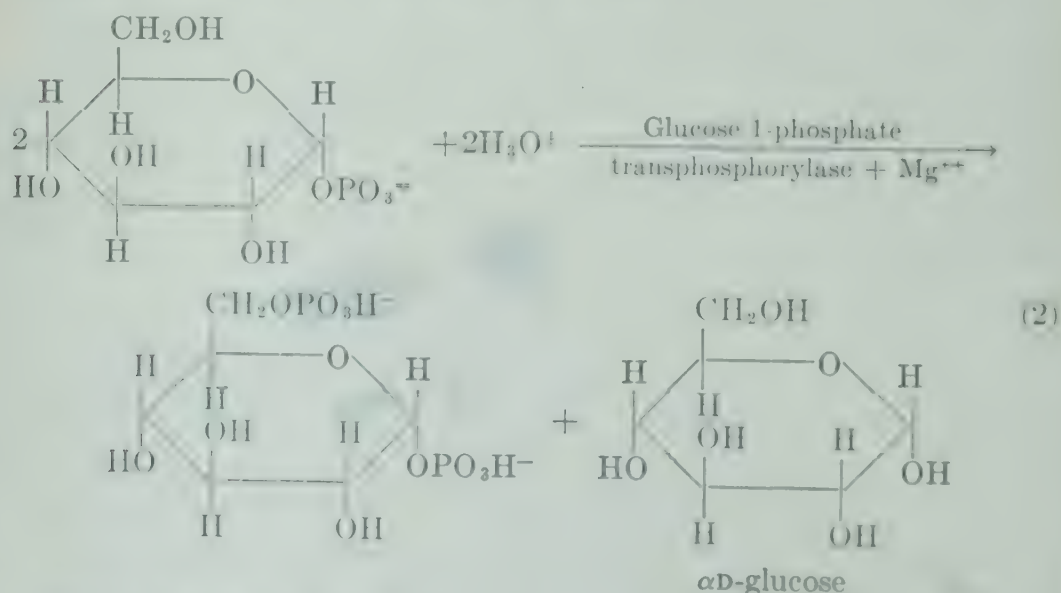
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it is conceivable that the intracellular polysaccharide might be composed of fructose rather than glucose units. Such a carbohydrate could be converted to fructose phosphates, and from these, glucose phosphates might be derived by a reversal of the reactions shown. Whatever the nature of the stored cellular polysaccharide it should be dissimilated essentially as illustrated except for the point at which it is introduced into the metabolic network. Complex extracellular carbohydrates may be fermented of course, but probably are affected differently in the first stages of their utilization than are the intracellular carbohydrates (see fig. 85).

Phosphoglucotransferase (also known as phosphoglucomutase) catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate. This reaction depends on the presence of glucose-1,6-diphosphate, and the transfer leaves glucose-6-phosphate as a product of the reaction which is indicated as follows:



The glucose-1,6-phosphate is derived from two molecules of glucose-1-phosphate by means of the transfer reaction:



When glucose itself is the initial substrate for fermentation, a phos-

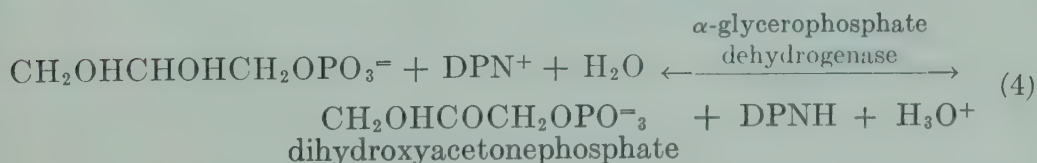
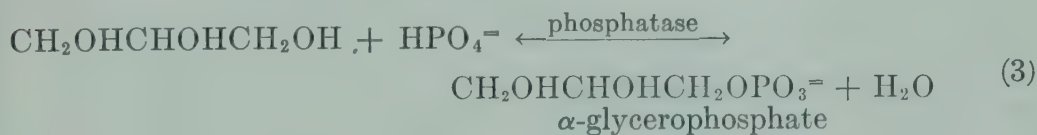


phorylation is required. The reaction is essentially irreversible and is catalyzed by hexokinase, an enzyme of broad distribution. During the process ATP (adenosinetriphosphate) is converted to ADP (adenosinediphosphate). This conversion of ATP to ADP results in a sharp drop of about 10,500 calories in free energy. Of this energy 3,000 calories is utilized in phosphorylating the glucose, but the remainder is not directly utilized except perhaps to drive the reaction far toward completion. Thus the high level chemical energy required at this step must be made available at some other step in order for the system to be workable.

Hexoisomerase mediates the next known reaction, serving to alter the geometric configuration of the hexosephosphate. This enzyme is also called phosphohexoisomerase and oxoisomerase. Fructose-6-phosphate resulting from the isomerization is further phosphorylated in the one position to yield fructose-1,6-phosphate. The reversibility of this particular reaction is somewhat in doubt. Once again a source of high energy phosphate is necessary, making a total of two such phosphate molecules consumed in the fermentation of one molecule of glucose.

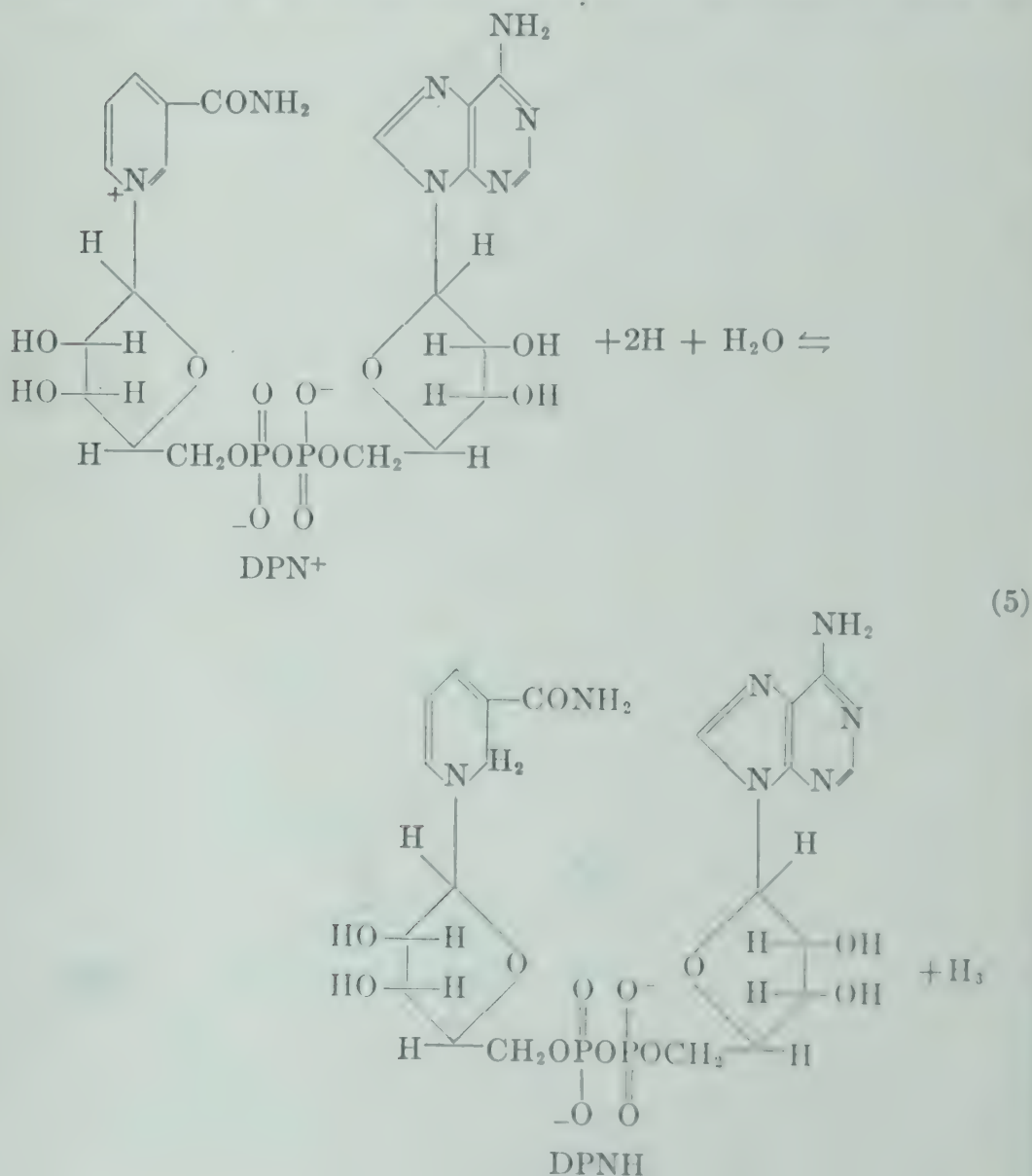
Aldolase splits fructose-1,6-diphosphate into two fragments of three carbon atoms by a reaction that is somewhat reversible. The enzyme is known to occur in some of the bacteria possessing anaerobic mechanisms and may require a metal activator. One of the reaction products, dihydroxyacetone phosphate, is not otherwise metabolized but is enzymatically converted into the other fragment, 3-phosphoglyceraldehyde. This arrangement permits a more complete utilization of the original carbohydrate than would be possible if dihydroxyacetone phosphate constituted an end product of fermentation. Up to this point structures, each containing six carbon atoms, have been involved as reactants except for the polysaccharides and other fermentable non-hexose carbohydrates listed in Figures 84 and 85. However, from this stage through the appearance of pyruvate the information summarized in Figure 83 indicates the participation of three carbon compounds to the exclusion of compounds of other chain lengths.

It is worthy of note that those organisms capable of metabolizing glycerol probably do so through dihydroxyacetone phosphate. The mechanism is presumed to involve the reactions



Both steps are enzymatic and reversible.  $\alpha$ -Glycerophosphate shows a small change in free energy on hydrolysis and as a result is formed to some extent without requiring the participation of the typical phosphorylating compounds having a high free energy of hydrolysis. Once the dihydroxyacetonephosphate is formed the usual mechanisms would be employed for further metabolic changes.

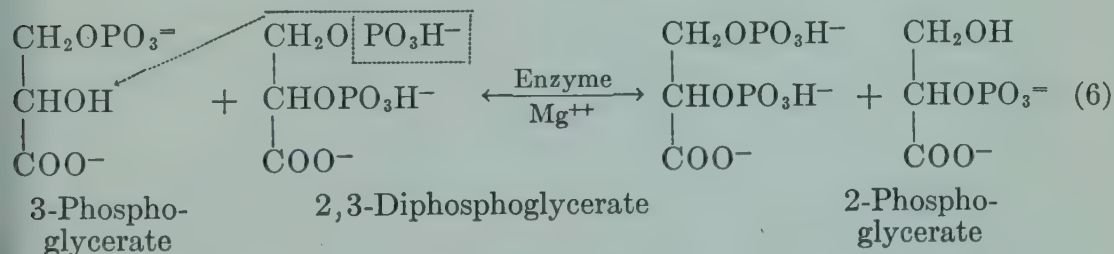
The conversion of 3-phosphoglyceraldehyde to 1,3-diphosphoglycerate is a complex process involving both phosphorylation and oxidation. Yet only one enzyme is necessary and this acts to catalyze the oxidation. It is quite possible that the phosphate being added to the 1-position reacts with



the 3-phosphoglyceraldehyde on the surface of this same enzyme making possible a simultaneous phosphorylation and oxidation. On the other hand, the phosphate may combine in a spontaneous reaction with 3-phosphoglyceraldehyde to form the theoretically possible compound 1,3-diphosphoglyceraldehyde. The latter substance would in all certainty not be stable and would either lose phosphate or undergo oxidation. Coenzyme I (diphosphopyridine nucleotide,  $\text{DPN}^+$ ) is essential for the oxidation to 1,3-diphosphoglycerate and is itself reduced. The  $\text{DPNH}$  thus formed must be reoxidized elsewhere in the system or fermentation would soon cease because of the depletion of suitable oxidizing agent at this point. Reduced  $\text{DPN}^+$  is written as  $\text{DPNH}$  because only one hydrogen atom is actually taken up by the  $\text{DPN}^+$  although two electrons are acquired in the reduction. Reaction (5) indicates the structures of both forms of coenzyme I and shows that the second hydrogen atom involved in the reduction appears as a hydrogen ion.

During the course of the oxidative phosphorylation just outlined, the readily available free energy rises sharply. The free energy of hydrolysis of one phosphate group is higher for 1,3-diphosphoglycerate than for ATP. Therefore, this energy might be made available to the organism for other uses by way of ATP. This possibility is actually realized in the presence of phosphoglycerickinase (phosphokinase) which transfers phosphate from the one position of 1,3-diphosphoglycerate to ADP. Since two units of three carbon atoms each undergo this reaction for each glucose molecule fermented, two ATP molecules are formed replacing those utilized in preceding reactions.

The enzyme known as phosphoglycerotransferase or phosphoglyceromutase transfers phosphate from the three position to the two position of glycerate. Apparently this reaction is somewhat analogous to that occurring with the glucose phosphates (reaction (1)) and is written as,



In the presence of magnesium, enolase dehydrates the 2-phosphoglycerate which has a relatively low free energy of hydrolysis into phosphoenolpyruvate which has a high free energy of hydrolysis. The phosphoenolpyruvate is the second type of high energy molecule formed during fermentation without the concurrent disappearance of some other high energy



compound. In this particular case the potentially available energy is converted to a usable form by means of phosphopyruvickinase which transfers the phosphate group from the phosphoenolpyruvate to ADP to yield ATP and enolpyruvate. The latter compound no longer has the high free energy which is now resident in the ATP. Since two molecules undergo this reaction for each glucose molecule fermented, two new molecules of ATP are generated at this stage.

Enolpyruvate spontaneously yields the keto form of pyruvate, the equilibrium being far in the direction of ordinary pyruvate. Indeed it would appear difficult to reverse these last reactions and to convert pyruvate to phosphoenolpyruvate. Presumably the enzyme phosphopyruvickinase does not alter the position of the equilibrium between enolpyruvate and phosphoenolpyruvate. A large excess of ATP would aid such a reversal by tending to withdraw any enolpyruvate present. However, phosphoenolpyruvate has a free energy of hydrolysis of about 4000 calories more than ATP, and this extra energy would have the effect of further reducing the tendency for phosphorylation of enolpyruvate with ATP as the source of phosphate. As a final result of the presence of the two reactions each having a strong tendency to go forward, it does not seem possible to accumulate much phosphoenolpyruvate once the fermentation has proceeded to the stage of pyruvate formation.

This effective irreversibility may be important in fermentation for a rather complex reason. Assuming for purposes of illustration that lactate resulting from the reduction of pyruvate is the end product of fermentation, this compound will accumulate in the medium up to rather high concentrations after the culture has metabolized a large quantity of carbohydrate. Inasmuch as lactate might tend to yield pyruvate by reversal of the reaction with lactic dehydrogenase, then the entire reaction sequence could be reversed or, stated more properly, brought to a halt before the fermentation had proceeded very far. In other words, the nature of the equilibria between phosphoenolpyruvate, enolpyruvate, and pyruvate may actually allow for a more prolonged fermentation in a limited or closed system than would be possible otherwise.

Pyruvate is reduced to lactate by DPNH in the presence of lactic dehydrogenase. In this way pyruvate acts as a hydrogen acceptor and regenerates the  $\text{DPN}^+$  required for the oxidative phosphorylation of 3-phosphoglyceraldehyde. If the  $\text{DPN}^+$  is all kept in the reduced form by any device, the fermentation ceases.

Anaerobic metabolism by bacteria does not lead universally to lactate as a final product and many of the other end products which have been observed are listed in Table 48, page 521. However, the energetics of these systems are essentially the same as for the scheme of Figure S3. The overall

process may be summarized as shown in Table 47. Only a comparatively small net number of high energy compounds are formed during the fermentation of one unit of carbohydrate. Therefore, a large quantity of substrate must be dissimilated to provide energy for the various needs of the organism.

The polysaccharides have stored in them free energy which if released by hydrolysis would be lost to the economy of the organism. When such compounds serve as intracellular substrates this energy is conserved by the utilization of a phosphorylase reaction in place of a hydrolysis reaction. The resulting glucose-1-phosphate is subsequently converted to glucose-6-phosphate. While the formation of the latter substance from glucose requires ATP, its formation from intracellular polysaccharide does not. To this extent the polymeric substrate is more efficiently utilized than the monomeric substrate. Since the energy contained in the polysaccharide

TABLE 47

*A summary of the fermentation of carbohydrates to lactate*

The letter  $n$  denotes the number of hexose units in the intracellular polysaccharide molecule.

SUBSTRATE 1 MOLECULE OF	END PRODUCT	ATP USED	ATP FORMED	ATP GAINED	DPN <sup>+</sup> NET CHANGE	HPO <sub>4</sub> <sup>-</sup> CONSUMED
Glucose . . . . .	2 lactate	2	4	2	0	2
(C <sub>6</sub> H <sub>10</sub> O <sub>5</sub> ) $n$ . . . . .	2 $n$ lactate	1 $n$	4 $n$	3 $n$	0	3 $n$

must be somehow supplied during the original synthesis, the apparent advantage of this type of substrate is lost. This point will be enlarged upon later.

Inorganic phosphate is taken up by the organism in an amount equal to that required for the net synthesis of ATP. That the assimilated phosphate finds its way into ATP has been demonstrated by tracer studies. If phosphate is completely excluded from a medium, fermentation is retarded although in actual living systems much of the phosphate utilized is restored to the medium later on. This restoration or phosphate turnover results from the participation of ATP in processes requiring energy leading to the reaction



Not all ATP reacts in this way, however, and a part of the phosphate enters into cellular materials like nucleic acids and phospholipids. With such a net gain in the phosphorus content of the organisms there is a net disappearance of phosphate from the medium. Consequently, when no



phosphate is supplied in the medium, growth cannot be initiated, and the metabolic activity of the organism is much impaired.

The mechanism of fermentation which has been discussed is commonly called the *Embden-Meyerhof* pathway after two investigators whose fundamental discoveries led to its elaboration. It was believed for a long time that the carbohydrate metabolism of all organisms proceeded by this mechanism with species differences appearing in steps beyond the formation of pyruvate. While many bacteria clearly do ferment carbohydrates

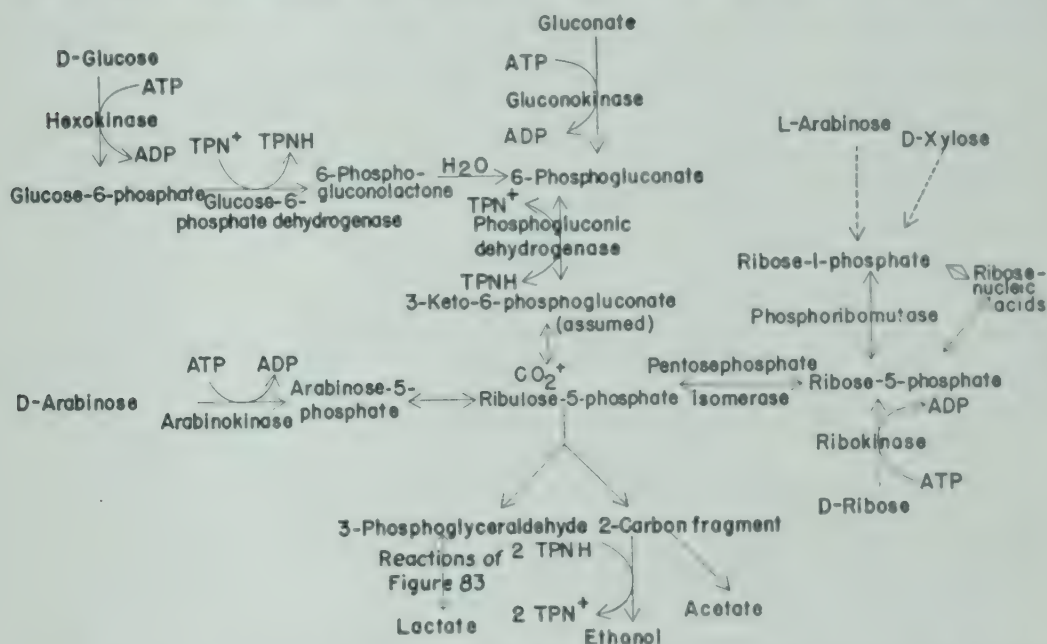


FIG. 84. Fermentation of pentoses and of glucose by way of the pentosephosphate pathway. Several debatable or uncertain steps are discussed in the text. TPN<sup>+</sup> is triphosphopyridine nucleotide or coenzyme II. Its structure resembles that of DPN<sup>+</sup> as shown in reaction (5) except that an additional phosphate group is present.

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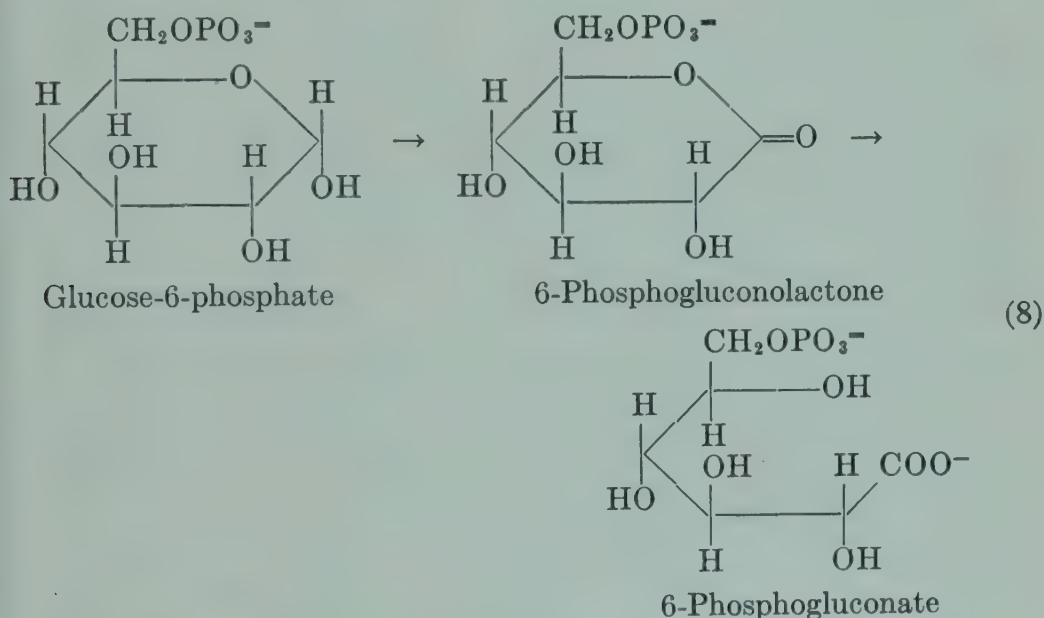
by the Embden-Meyerhof scheme, others possess an alternative pathway and possibly may utilize either or both. In still other bacterial species belonging to the group called heterofermenters (*Leuconostoc*, for example) the classical Embden-Meyerhof mechanism cannot be involved in carbohydrate fermentation because these organisms do not contain the key enzyme aldolase for splitting fructose-1,6-diphosphate into 3-phosphoglyceraldehyde and dihydroxyacetonephosphate. Furthermore, pentoses may serve instead of hexoses for bacterial fermentations and with these carbohydrates the usual scheme for dissimilation of six carbon atom compounds cannot be applied unless a means were available for the prior conversion of the pentose into a hexose derivative.

Figure 84 summarizes the results of investigations bearing on this second



pathway. It will be observed that the hexoses may all pass through this system by virtue of prior conversion to glucose-6-phosphate. The specific reactions carrying out such conversions are shown in Figure 85 and will be discussed later. In addition, the pentoses may be utilized as shown although there are many points of uncertainty. Eventually the process leads to a triosephosphate which is handled by the classical mechanism. A two carbon unit is formed at the same time as the triosephosphate and travels a different route.

The steps shown in Figure 84 which have been demonstrated may be described somewhat more fully. Starting with glucose-6-phosphate the dehydrogenase acting on this substrate catalyzes an oxidation by means of coenzyme II ( $\text{TPN}^+$ ). The first product formed is a lactone whose ring structure slowly opens upon hydrolysis yielding 6-phosphogluconate. The details of the reaction of the lactone are not yet understood; however, the overall process may be written:



6-Phosphogluconate may also arise by phosphorylation of gluconate in the presence of ATP and a specific kinase. Whatever its origin, this intermediate is further oxidized in the presence of  $\text{TPN}^+$  and phosphogluconic dehydrogenase. The first product, which to date has been shown to appear, is ribulose-5-phosphate with the accompanying evolution of carbon dioxide. Since this overall process is quite complex, at least one intermediate is postulated, 3-keto-6-phosphogluconate formed by oxidation at position three. Decarboxylation then occurs. As has been shown by labeling experiments with isotopic tracers, the carbon dioxide comes from the position which was formerly the aldehyde group of the glucose.

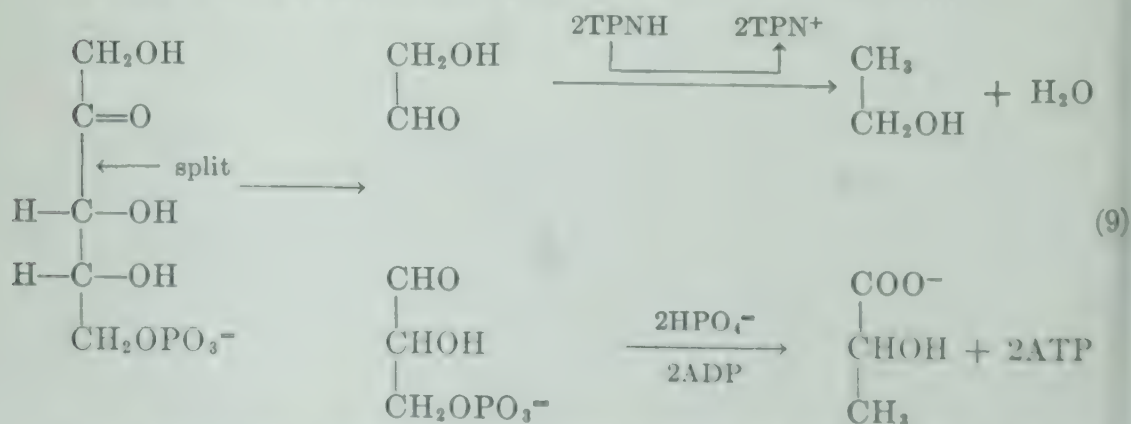
At this point the fermentation of pentoses enters into the reaction pattern.

D-Ribose is phosphorylated by means of a typical reaction to ribose-5-phosphate which is then isomerized to ribulose-5-phosphate or employed in the synthesis of nucleic acids. Furthermore, ribose-5-phosphate may be formed from ribose-1-phosphate by means of phosphoribomutase which is probably a transferase similar to phosphoglucotransferase and acting with a coenzyme.

L-Arabinose and D-xylose are fermented usually after an adaptive enzyme has formed. It has been proposed that these two sugars are converted by some unknown means to ribose-1-phosphate. In view of a complete lack of evidence this hypothesis remains speculative. D-Arabinose, on the other hand, is definitely phosphorylated to arabinose-5-phosphate which is then converted reversibly to ribulose-5-phosphate as shown in Figure 84. Of these reactions involving pentoses and their phosphates, some have been studied with *Escherichia coli* and others with *Leuconostoc* species.

Once the pentose phosphates are formed, a split into two and three carbon fragments occurs. There is a difference of opinion as to which pentose derivative is the one actually split. From a purely theoretical point of view the ribulose-5-phosphate seems the most likely candidate. In any event a triosephosphate is formed, but it too is of uncertain nature. Since it is probably either 3-phosphoglyceraldehyde or dihydroxyacetone phosphate, which exist in an equilibrium with each other, subsequent reactions would appear to follow the course outlined in Figure 83 for these compounds.

The nature of the two carbon fragment is unknown but it is the source of acetate or ethanol with other end products as additional possibilities. When glucose is the initial substrate this overall pathway reduces two  $\text{TPN}^+$  which as a coenzyme occurs only in trace amounts in organisms. It is suggested, therefore, that the two carbon fragment is reduced to ethanol with the concurrent oxidation of  $\text{TPNH}$  in the presence of appropriate enzymes. A purely hypothetical process is shown in reaction (9).



These theoretically possible reactions will account for the products, but the

formation of glycolicaldehyde and various key intermediate reactions have yet to be demonstrated. The orientation of carbon in the products is as shown in (9). Tracer studies with *Leuconostoc* species indicate that the carbon dioxide comes from carbon-1 of glucose and that the other carbon atoms appear in order as the methyl and hydroxyl substituted carbons of ethanol and the carboxyl, second position, and methyl carbons of lactate. If pentose is fermented as shown in Figure 84,  $\text{TPN}^+$  is not reduced since there is no occasion to form the highly oxidized substance, carbon dioxide. Therefore, the resulting absence of  $\text{TPNH}$  would prevent the formation of ethanol, and thus only acetate would be formed from the two carbon fragment.

Finally, it will be observed that when either hexose or pentose is fermented by the mechanism of Figure 84, one molecule of ATP is required. As the process is now understood, two molecules of ATP are generated representing a net gain of one. Since this seems to be the only free energy gained at a high level and in a usable form, a large quantity of substrate must be metabolized for growth and synthetic processes. Indeed this pathway is even less efficient than the Embden-Meyerhof mechanism which, of course, may not be available for pentose fermentation. The pathway outlined in Figure 84 through pentose phosphate would provide the same net quantity of useful high energy compounds for both hexose and pentose. In the case of *Leuconostoc mesenteroides* this relationship has been demonstrated since the net growth is the same per mole of glucose as it is per mole of arabinose.

Actually a good deal of effort has been expended in studying the reversibility of the reactions existing between glucose-6-phosphate and ribulose-5-phosphate. To date 6-phosphogluconate, one of the proposed intermediates, has not been transformed experimentally into glucose-6-phosphate, but further studies undoubtedly will be attempted. The problem is of fundamental importance for two reasons. First, such reactions would permit the metabolism of pentoses to go by way of the Embden-Meyerhof pathway in those organisms which can utilize that system. Moreover, this process involves fixation of carbon dioxide and might thus be valuable for purely synthetic purposes.

The foregoing discussion covers the known anaerobic transformations in the dissimilation of glucose to pyruvate. Although this phase of the work on metabolism is as well understood as any, a number of gaps and deficiencies have been noted. Probably there are many others not yet envisioned, and there may well exist whole pathways still unsuspected. Indeed as work progresses with bacteria new metabolic processes are beginning to appear, hinting at the existence of variations among bacterial species in their intermediate metabolism of carbohydrates.

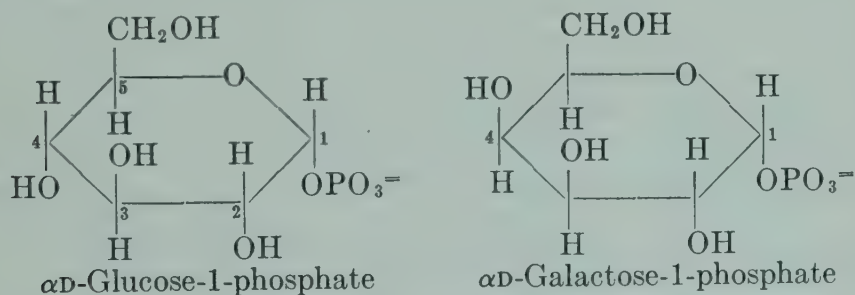




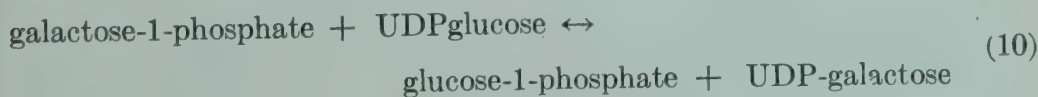
expect that fructose as an external substrate might be readily metabolized since fructose derivatives appear in the Embden-Meyerhof pathway. This expectation is met, for fructose is phosphorylated by means of hexokinase and ATP to fructose-6-phosphate. This last substance may be either broken down or converted to polysaccharide by reactions which have been shown to be reversible.

Mannose commonly is attacked by bacteria. As a first step it apparently is phosphorylated in the 6-position with ATP supplying the phosphate and is then isomerized by means of an enzyme into either glucose-6-phosphate or fructose-6-phosphate. At the moment it is not possible to tell which event occurs since the enzyme preparations studied contain hexoisomerase which leads to an equilibrium mixture of the three sugar phosphates, mannose-6-phosphate, glucose-6-phosphate, and fructose-6-phosphate. Mannose-1-phosphate and mannose-1,6-diphosphate probably also are formed in the same way as the corresponding glucose derivatives. However, the function of these compounds of mannose is not clear. Similar fructose derivatives are known, and like the corresponding mannose derivative fructose-1-phosphate has not been fitted into the picture. On the other hand, fructose-1,6-diphosphate is a key intermediate compound.

Galactose in the presence of ATP is acted upon by a widely distributed enzyme, galactokinase. Instead of the 6-phosphate produced by the ordinary hexokinase acting on glucose, fructose, and mannose, galactokinase catalyzes the formation of the 1-phosphate of galactose. Galactose-1-phosphate differs from glucose-1-phosphate in the orientation of the hydroxyl group on the fourth carbon atom:

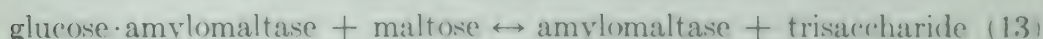
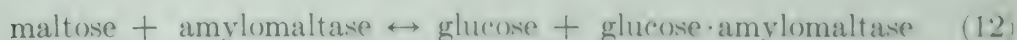


A mechanism for the reversible conversion of one into the other has been discovered. It involves the coenzyme uridinediphosphoglucose (UDP glucose) which presumably functions in the following two-stage system catalyzed by phosphogalactoisomerase:



Experimentally these reactions have not been separated. Nor has reaction (10) been definitely established, but reaction (11) seems to be the step at which the Walden inversion occurs.<sup>3</sup>

Turning now to the dissimilation of disaccharides a series of interesting reactions is known. Most bacteria are capable of attacking one or more disaccharides frequently, if not always, employing adaptive enzymes. Hydrolysis of disaccharides liberates free energy which may be utilized in the synthesis of polysaccharides by bacteria. *Escherichia coli* produces amylomaltase which catalyzes the conversion of maltose into a polysaccharide with the liberation of glucose. Neither phosphate nor any known high energy compound intervenes in this reaction which is reversible. The size of the polymeric polysaccharide synthesized depends upon the concentrations of maltose and of the glucose formed. If during the synthesis, glucose is removed the resulting polysaccharide is of high molecular weight and resembles starch. The reaction involves a mechanism called transglucosidation and may be represented by the equations,



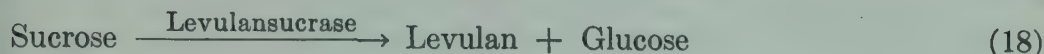
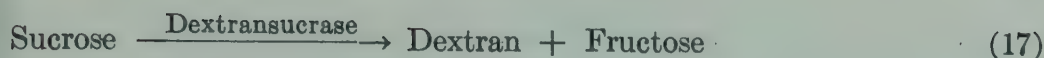
According to this scheme the energy in the disaccharide is preserved in the glucose-enzyme complex and transferred from it into the trisaccharide. Subsequent steps increase the size of the product, the glucose concentration controlling the extent of the process. Polysaccharide formed in this way from maltose is similar to that formed from glucose through the phosphorylation process although it may not be identical. At any rate, the polysaccharide is a readily utilizable source for the formation of glucose-1-phosphate by means of the typical phosphorylase reaction.

By a very similar mechanism sucrose may be utilized for the formation of the same sort of polysaccharide. The enzyme involved is amylosucrase and the monosaccharide released during the polysaccharide synthesis is fructose. Sucrose may also be acted upon in other ways as is shown in Figure 85. The known reactions are listed:



<sup>3</sup> A Walden inversion is a reaction producing the opposite steric configuration about the atom in question. In other words, the asymmetry of the atom to which the reacting group is attached is reversed.





Of these overall processes, some of which may involve complicated series of intermediate reactions, only (16) involves phosphate in any way. In this reversible reaction the energy of hydrolysis of sucrose is transferred to glucose-1-phosphate. In reaction (15) reversibility is unlikely and has not been studied in the remaining cases. Fructose is an end product in all the reactions but (18) where the fructose is utilized directly and glucose is set free.

The dextran formed by the dextransucrase of *Leuconostoc mesenteroides* is an extracellular polysaccharide made up of glucose units combined in 1,6-glucosidic linkages. This end-to-end combination of the monosaccharide molecules is in contrast to the 1,4-linkage characteristic of amylose and the intracellular polysaccharide discussed previously. Different enzymes are required for the formation and decomposition of these two types of linkage.

While enzymes may exist for carrying out equivalent reactions with disaccharides other than those discussed, little search has been made for them. Raffinose, a trisaccharide of galactose, fructose, and glucose, definitely undergoes some of the same types of reactions shown for sucrose.

The various polysaccharides formed by bacteria presumably can be broken down by bacterial species. One would expect that levulan (or levan), for example, should be converted to fructose and thence into the metabolic network as the needs of the organism may require. However, neither levulan nor dextran is definitely known to be utilized. Cellulose, on the other hand, has been more intensively studied. Some bacterial species are able to degrade the plant celluloses to glucose, presumably passing through cellobiose, by means of cellulases and cellobiase. In other cases cellobiose and even glucose may not be intermediates in the degradation of cellulose.

The preservation of the free energy of hydrolysis of cellulose, cellobiose, levulan, dextrans, and other polysaccharides acted upon by amylases has not been discussed. If the hydrolytic products are the simple monosaccharides, all of this potentially useful energy may be lost unless some form of coupling mechanism can be established. The inefficiencies of the situation written into Figure 85 are quite serious because ATP is needed to phosphorylate each monosaccharide formed and the free energy demands thus become high. However, it is possible that the energy lost during hydrolysis is employed in some indirect way in driving other reactions. It also may be that under normal conditions derivatives of monosaccharides are

formed that can be metabolized without the initial phosphorylation with ATP. In this connection a phosphorylation like that discussed for the intracellular polysaccharide would be effective except for those extracellular carbohydrates of large size which do not penetrate organisms.

*Acetobacter xylinum* synthesizes a cellulose much like that of the higher plants. Since the preferred substrate for this synthesis is fructose, the process must at least pass through a glucose derivative in order to provide the basic glucose units making up the cellulose molecule. Glucose, galactose, and the natural starch-like intracellular polysaccharide may also act as sources of raw material for the synthesis of cellulose. Therefore, this synthesis would seem to involve one or more of the intermediates normal to the metabolism of this bacterium.

Added carbohydrates like glycogen, amylopectin, amylose, and the dextrans may be converted by amylases to maltose and metabolized by the normal pathways for glucose utilization. In this degradation the free energy of hydrolysis does not seem to be directly utilized in any way except for that fraction in maltose which may pass directly into an intracellular polysaccharide. The amylases differ in their specificities attacking different linkages in the extracellular substrates. In amylose only the  $\alpha$ -1,4-linkage exists, and one enzyme can cleave it. The hydrolysis of the branched compounds, glycogen and amylopectin, needs this same amylase plus another enzyme which can attack the branches. Organisms attacking the polysaccharides either possess the necessary complement of enzymes constitutively or form the enzymes adaptively in the presence of the specific substrate.

The strange process of conversion of dextrin to dextran by *Acetobacter viscosum* and *Acetobacter capsulatum* involves an enzyme called dextran-dextrinase. Certainly a change from 1,4-dextrin to 1,6-dextran linkages occurs, but the evidence does not permit speculation about the mechanism. Not all the known dextrans are the same, for those produced by means of dextransucrase by *Leuconostoc mesenteroides* and *Leuconostoc dextranicum* are branched and straight, respectively. Evidently these materials are susceptible to enzymatic degradation since on standing the cultures of organisms forming dextrans can reduce the viscosity due to the presence of this carbohydrate.

The metabolism of carbohydrates conceivably might lead to the formation of any of a large number of compounds as end products. The known products of bacterial fermentations are listed in Table 48, and others probably remain to be discovered. It must be made clear that all of these substances do not result from the metabolic activity of any one species although it is commonplace for two or more to accumulate. As may be inferred from the length of this list the fate of pyruvate may be quite complex.



Fermentation end products now will be discussed briefly from the point of view of their origin. Each compound will be treated separately except for carbon dioxide, water, and hydrogen which will be mentioned wherever they appear in connection with the other products.

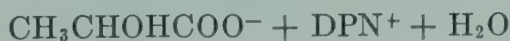
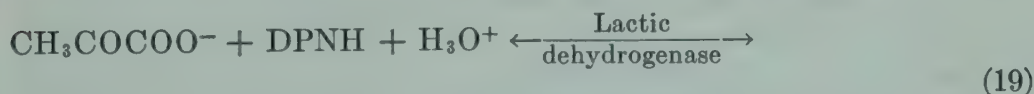
TABLE 48

*The end products of bacterial fermentations with a common intermediate of pyruvate*

In certain instances a number of these compounds can be intermediates, but are known to accumulate under particular conditions of fermentation.

Lactate	Ethanol	Propanol
Acetate	Succinate	Butyrate
Formate	Biacetyl	Butanol
Hydrogen	Acetoin	Acetone
Carbon dioxide	Butylene glycol	iso-Propanol
Water	Propionate	Trimethylene glycol

Lactate is formed by many bacteria and is the chief end product of fermentation by the so-called lactic acid bacteria. These organisms are classified into two categories known as *homofermentative* (homolactics) and *heterofermentative* bacteria. The first group produces lactate in high yield with no more than a minor accumulation of other products.



By providing a mechanism for the oxidation of reduced  $\text{DPN}^+$  the reversible reduction of pyruvate to lactate fulfills a basic requirement in the anaerobic processes that utilize pyruvate. In all of these cases some sort of a hydrogen acceptor is essential for the maintenance of the catalytic action of  $\text{DPN}^+$  or  $\text{TPN}^+$ , and this requirement influences the nature of the end products resulting from fermentation. During the dissimilation of hexoses when the pyruvate is reduced according to reaction (19), no products other than lactate are formed. Apparently this pathway is the one employed by homofermentative species of the genera *Lactobacillus*, *Streptococcus*, *Bacillus*.

The heterolactics form lactate plus appreciable quantities of other compounds. One mechanism employed by these organisms may be that of Figure 84 in which hexose is degraded through the pentosephosphate pathway to lactate, carbon dioxide, and ethanol and to traces of other compounds. The information on this system has already been discussed. An-



other possible process involves a dismutation of pyruvate to lactate and acetate:



This reaction clearly is complex, requiring more than one enzyme and involving more than one step. A number of species are able to dispose of added pyruvate as shown while *Staphylococcus aureus* and other species are able to reverse reaction (20). However, the extent to which this process plays a part in normal fermentation may be challenged, for no provision is made for the oxidation of DPNH. It should be recalled that this is necessary in maintaining the limited supply of the oxidized form of this coenzyme. Therefore, other hydrogen acceptors must be available in lieu of pyruvate, or a source of extraneous pyruvate must be added, otherwise reaction (20) merely serves as an exchange mechanism. ATP, cocarboxylase, and possibly still another cofactor may be involved in the process as carried out by the several species concerned.

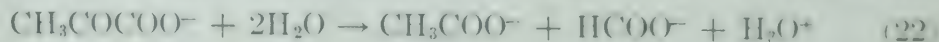
Rather oddly, different bacteria produce different isomers of lactate. *Streptococcus lactis* and *Lactobacillus delbruckii* yield D(-)-lactate, *Lactobacillus leishmannii* and *Leuconostoc mesenteroides* produce L(+)-lactate, and *Lactobacillus pentoaceticus*, a racemic mixture. These differences might lead one to suspect that the various mechanisms of lactate formation are even more complex than is now generally understood.

Acetate is formed as in reaction (20) and from pentoses and perhaps hexoses by way of the two carbon fragment derived from ribulose-5-phosphate (Figure 84). In addition, *Clostridium thermoaceticum* and *Butyribacterium rettgeri* are able to reduce carbon dioxide to acetate.



This mechanism and its relative importance are not understood, nor is the reducing agent (called simply H) known except that *Clostridium aceticum* is apparently able to use molecular hydrogen for the reaction.

*Escherichia coli* among others cleaves pyruvate somewhat as follows:



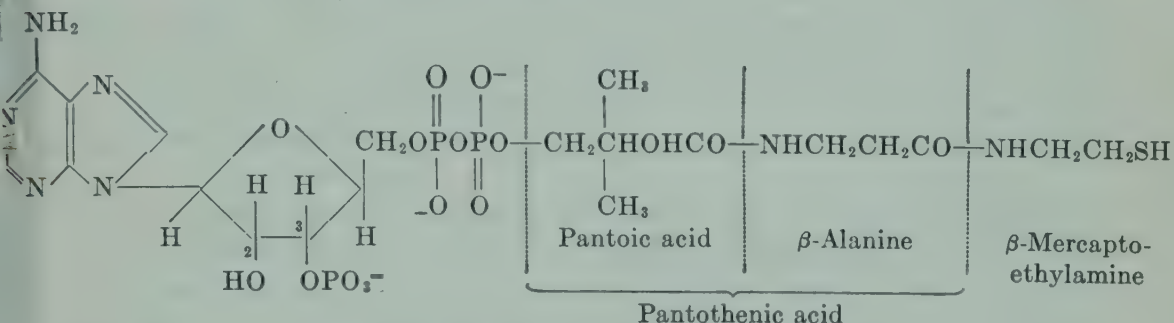
Phosphate, manganous ions, and cocarboxylase are required. This reaction is surprising in that a high energy phosphate is formed as an intermediate which is capable of phosphorylating ADP. It has been suggested that water is not a reactant but that instead the reaction is a phosphoroclastic one.



The brackets indicate that the indicated compound does not seem to be acetyl phosphate although it is very similar in many respects. More recent observations on the nature of the so-called active acetate indicate that this material is an acetyl derivative of coenzyme A instead of being acetyl phosphate. It therefore seems logical to replace reaction (23) with the enzymatic reactions (25) and (26):



which may then be followed by (24) yielding the high energy phosphate. Coenzyme A is tentatively represented as



The phosphate on position 3 of ribose may actually be on position 2. Coenzyme A seems to be acetylated on the sulfur atom. Hence this acetylation step could provide a hydrogen atom from the coenzyme A for the reduction of carbon dioxide, and the second electron would be obtained from the pyruvate upon decarboxylation. Acetylcoenzyme A hydrolyzes with a large decrease in free energy and as a result has been classified as a high energy compound. This substance is split by phosphate in the presence of phosphotransacetylase which is found in bacteria. The acetylphosphate formed can readily phosphorylate ADP.

Such a degradation of pyruvate as reactions (25), (26), and (24), instead of the use of the pyruvate as a hydrogen acceptor, again raises the problem of the nature and availability of suitable hydrogen acceptors unless the carbon dioxide can be employed as in reaction (21). However, *Escherichia coli* does not seem to carry out this latter process although other species can. It has been suggested that reactions of the above series or related ones play a role in the dismutation process (20).

A very similar cleavage is brought about by *Clostridium butylicum*



However, unlike reaction (23) carbon dioxide and hydrogen are produced without the mediation of formate as an intermediate. High energy phos-



phate can be obtained by (26) and (24) and provides a greater energy return than most other pathways of fermentation.

Formate has its origin either as shown above in reaction (23) or by synthesis from hydrogen and carbon dioxide. The overall reaction in the latter case is



This reversible process seems to be complex and may require the participation of more than one enzyme. One would expect little formate from (28) except in a closed system which would prevent the escape of hydrogen. Thus the reaction would not ordinarily occur in nature. Many species of bacteria are able to catalyze this reaction, but most actually use it in the reverse direction for the formation of hydrogen. This gas is a rather common product of bacterial fermentations and apparently has its origin in reaction (27) or (28). In either case it appears that the production of hydrogen depends upon the conversion of pyruvate to a two carbon compound of high energy.

Ethanol is shown in Figure 84 to be a product of hexose fermentation via the pentose phosphate pathway. This hypothesis cannot be assessed at the moment in terms of quantitative relationships or mechanisms. Two species, *Termobacterium mobile* (*Pseudomonas lindneri*) and *Sarcina ventricula*, are rather unusual as bacteria go in their fermentation of glucose. The principal end products of fermentation by these species are carbon dioxide and ethanol produced by a mechanism resembling that of yeast. The results may be summarized by the following:

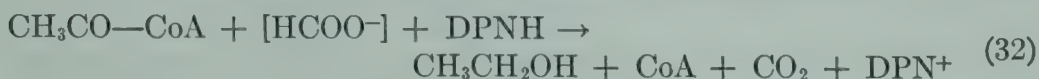


The enzyme carboxylase catalyzes the reaction and requires cocarboxylase for its activity. Although this mechanism readily accounts for ethanol production, it has not been possible to demonstrate carboxylase in those bacteria producing ethanol other than in the above two species. Furthermore, other organisms yield products in addition to carbon dioxide and ethanol suggesting that other mechanisms may be operating.

A possible precursor of ethanol is acetaldehyde which actually does accumulate in the presence of a trapping agent. However, the origin of the trapped aldehyde has not been clarified. The most promising theory for the formation of ethanol implicates acetylphosphate or more probably acetyl coenzyme A. This key substance could be formed by reaction (25) and subsequently reduced to ethanol. The necessary hydrogen donors are available in DPNH and formate both of which would be required by the

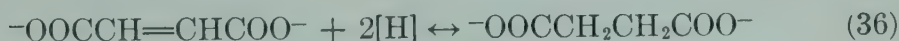
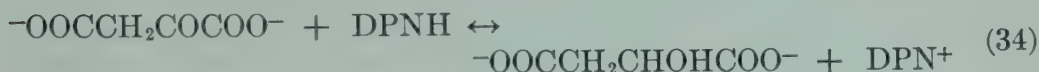
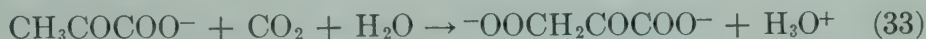


stoichiometry of the assumed reactions. Some data have been interpreted as indicating the existence of an active one carbon precursor for formate. If such a compound is formed momentarily it also would be of fundamental importance in ethanol formation according to the scheme



Both of these processes are complex and certainly would require more than one step for their completion. The high energy compound,  $\text{CH}_3\text{CO}-\text{CoA}$  formed from pyruvate would be consumed in the end products, but this disadvantage in the lowering of the free energy available is offset by the reoxidation of the DPNH which must take place in some manner if the cycle of metabolic activity is to be maintained. Many of the ideas applied to the production of ethanol can be extended to the appearance of other alcohols in fermentation.

A variety of bacteria produce succinate as a result of their fermentations. The appearance of succinate is matched quantitatively with the disappearance of carbon dioxide, and mechanisms have been proposed to explain a large body of such experimental observations. The most widely accepted hypothesis is called the Wood-Werkman reaction and may be outlined:

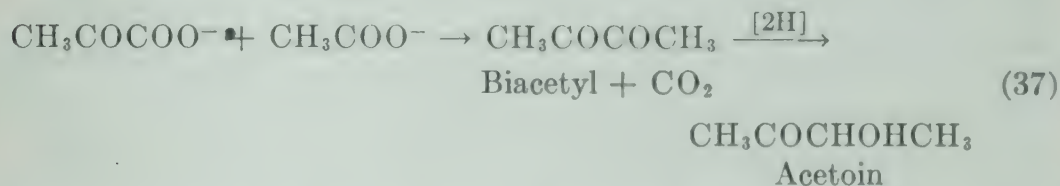


Reaction (33) is more complex than is indicated and recently has been investigated in connection with the general problems of photosynthesis and the fixation of carbon dioxide. See also the section on the mechanism of photosynthesis. The hydrogen donor in (36) has not been identified. The other steps are rather well understood individually and seem pertinent to the scheme since the participating enzymes have been characterized and can account for the necessary reactions. In addition, fumarate and malate accumulate during some of the fermentations that yield succinate. This process supplies a sufficiency of hydrogen acceptor, actually more than enough for the fermentation of each triosephosphate. Consequently it is possible that this process might occur in conjunction with some of the acetate reactions which do not elaborate a hydrogen acceptor.

Tracer studies with extracts of *Escherichia coli* have shown that not all

of the succinate formed by this organism can have its origin in reactions (33) to (36). It is definitely established that both *Escherichia coli* and *Aerobacter indologenes* convert acetate to succinate. The latter organism actually is able to yield succinate from glucose without any evidence of the participation of carbon dioxide. In spite of the probable importance of this process suitable explanations of its mechanism have not been brought forth.

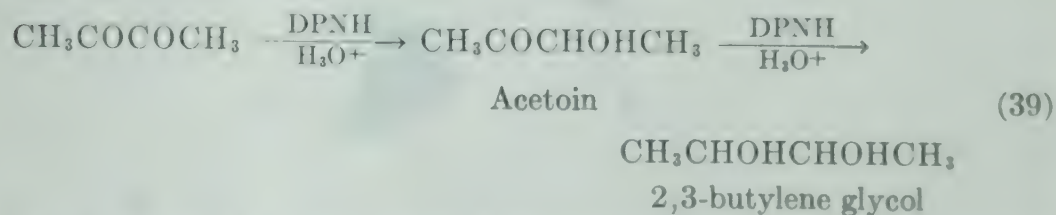
*Aeromonas hydrophila*, *Staphylococcus aureus*, *Clostridium acetobutylicum* and several species of *Aerobacter* and *Bacillus* synthesize biacetyl, 2,3-butylene glycol, or acetoin (acetylmethylcarbinol), or some combination of these. It is probable that organisms producing these compounds do not all employ the same mechanism. In this regard yeast and muscle tissue probably differ from *Aerobacter aerogenes*. In bacteria the overall system requires  $Mn^{++}$ , cocarboxylase, and perhaps phosphate although no intermediates containing phosphorus have been demonstrated as yet. It has been proposed that acetate may be condensed during the decarboxylation of pyruvate to yield biacetyl.



Apparently there are two enzymes in extracts catalyzing these reactions. The acetate might have its origin from pyruvate and appear as acetyl-CoA or be converted directly to acetylphosphate and subsequently to acetyl-CoA. The actual condensation then may take place between two of the intermediate molecules such as two acetyl-coenzyme A molecules. If acetyl-CoA from pyruvate does react with itself to form biacetyl, a hydrogen donor is needed, and this might be the active formate of reaction (31).



Further reduction by DPNH from earlier steps would then yield acetoin and finally butylene glycol.



As in the case of the lactic acid fermentation, different species of bacteria

form different optical isomers of 2,3-butylene glycol; all three of the possible isomers have been detected.

*Propionibacterium* and a few other species produce sizable quantities of propionate during their fermentations. The general nature of the process seems to be the same for the various bacteria and proceeds from pyruvate by way of succinate. Succinic decarboxylase acts reversibly on succinate to produce propionate and carbon dioxide.

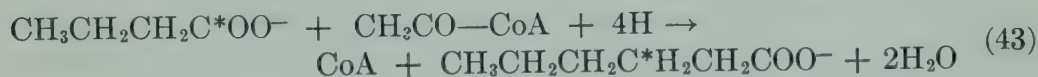


The formation of the succinate already has been discussed. *Aerobacter indologenes* converts both propionate and propionaldehyde to n-propanol when glucose is being fermented. The probable mechanism would seem to be like the one yielding ethanol and involves reduction of an acyl coenzyme A to the alcohol.

Several species of *Clostridium* form butyric and higher molecular weight acids. The latter are apparently formed by mechanisms of the types involved in acetate, propionate, and butyrate formation so they will not be considered separately. Butyrate itself is formed from acetate, apparently through acetyl-CoA and acetate:



The intermediate has evaded discovery to date. When carboxyl labeled acetate is introduced into an appropriate preparation acetyl-coenzyme A forms from it, and the butyrate which results may undergo a further and analogous reaction to elaborate caproate in which a tagged carboxyl appears in the  $\beta$  position.



This indicates the coupling of the carboxyl of the butyrate to the methyl group of the acetate molecule. Still longer even numbered carbon chain acids form in this way. Acids containing an odd number of carbons are produced starting with propionate which is combined with acetate. Hydrogen gas may supply the needed hydrogen atoms. The mediation of hydrogen carriers would be necessary, which also could be responsible for the reduction when they are present in the reduced state in sufficient quantities in the absence of a supply of hydrogen gas. See the discussion of lipid metabolism for a more detailed mechanism of the breakdown and synthesis of fatty acids.



Butanol is produced in rather large quantities by *Clostridium butylicum* which apparently converts acetate to butyrate and subsequently reduces the latter to the corresponding alcohol. Presumably the carboxyl group is reduced by the same mechanism that is used in other systems.

Several clostridia form acetone in fair yields with acetoacetate probably acting as an intermediate. The process is a branch from the butyrate mechanism utilizing the unknown intermediate formed in reaction (41):



The decarboxylase catalyzing (45) is formed only when the system is in an acid environment and has an acid range of optimum activity. Indeed, at pH values above 6.5 acetone is not elaborated. One would expect that acetone might serve as a hydrogen acceptor, and quantities of iso-propanol actually can be formed when acetone is present. Almost any hydrogen donor system might serve in this reduction.



Some *Aerobacter* and *Escherichia freundii* yield trimethyleneglycol as a fermentation product. However, this compound ( $\text{CH}_2\text{OHCH}_2\text{CH}_2\text{OH}$ ) is a surprising one bearing no close relationship to other known fermentation products and their intermediates. One might speculate as to whether oxaloacetate or malate is decarboxylated to yield the corresponding carbonyl or hydroxy propionates. Once formed, these compounds might be reduced to trimethylene glycol by known systems. Unfortunately, there seems to be no evidence on the matter.

At this point in the discussion it should be evident that fermentation leads to a diversity of end products and also that diversity of end products is customary for fermentations by single species of bacteria. This situation seems to have its origin in the fundamental requirement for hydrogen acceptors during fermentation. Since several different potential acceptors are present, more than one is involved in the usual case. Furthermore, the relative quantitative roles of the various equilibria may be altered strikingly by changes in conditions. The nature and concentration of fermentable substrates, the species and age of the bacteria, pH, salts, and the end products already present are among the factors controlling the nature and quantity of the end products formed at any given time. To the extent that these factors vary with time, it will be evident that the results of fermentation will also change with time.

Some typical examples of the variability may be cited from experiments with *Escherichia coli* and the homofermentative bacteria which character-

istically produce lactate in acid solution. The latter species give quantitative yields of lactate from glucose, but in neutral or slightly alkaline media the major products are acetate and formate. Acetoin is never formed during bacterial fermentations unless the pH is below 6.3, and succinate does not accumulate appreciably in cultures of *Propionibacterium* unless the pH is near 6.5 or higher. Even the concentration of carbon dioxide is important. *Escherichia coli* produces but little succinate when there is little carbon dioxide for fixation. It, therefore, becomes impossible to write balanced reactions for the complete fermentative process of the ordinary bacterium or even to write a set of overall reactions showing the products actually formed under all conditions.

Earlier it was suggested that bacteria assimilate and can store an intracellular reserve material under anaerobic conditions. Although of fundamental importance, knowledge of the mechanism of this assimilation is scanty. One must assume that substrate is dissimilated to the various end products in order to provide the energy needed in the synthetic reactions involved in assimilation. The manner in which this energy is employed and the compounds taken for use as synthetic units from the ordinary dissimilatory pathway and applied to synthetic purposes are quite unknown. Since the fermentation of hexose does not provide very much energy, the anaerobic assimilation yields only small quantities of reserve material when compared to the large amounts of small molecules formed as end products.

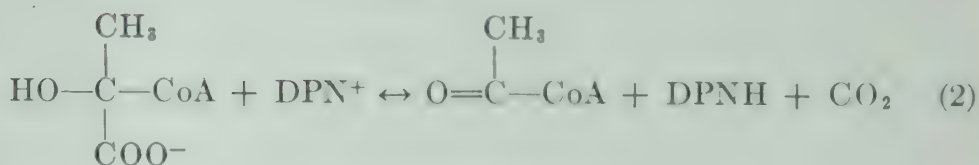
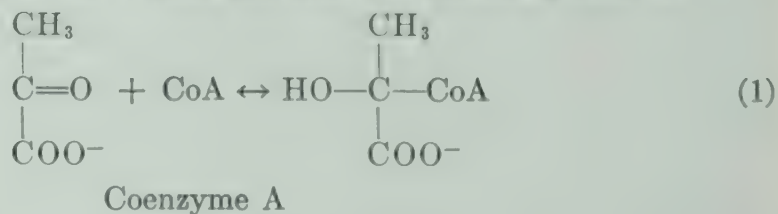
### *Aerobic Mechanisms*

Respiration involves a greater number of processes than fermentation and may be defined as including those operations for which molecular oxygen acts as the ultimate hydrogen acceptor. These aerobic pathways are complex, and a great deal of information has been collected which will be summarized herein. Attention will be focused on those systems which seem to be most widespread and quantitatively the most important in bacteria. The various reactions occurring at and immediately before the end processes of aerobic dissimilation are grouped together under the term *terminal respiration* and will be considered first.

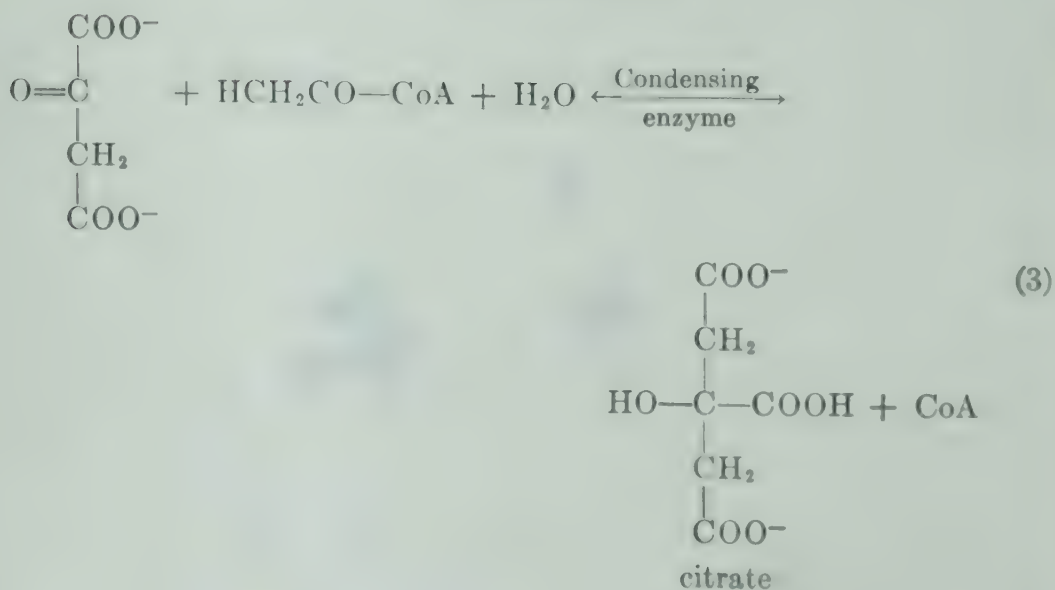
Fermentative processes elaborate pyruvate, acetate, and perhaps ethanol as primary products of dissimilation. These substances may be further degraded by means of anaerobic mechanisms. Pyruvate, especially, as has been outlined, may yield numerous end products. Pyruvate and acetate may also be formed in aerobic systems but unlike the case in anaerobic systems they undergo complete oxidation to water and carbon dioxide. Neglecting for the moment the problem of the origin of pyruvate, since two possible pathways have already been outlined in the discussion of fermentation, let us consider the complete oxidation of this compound.

Pyruvate may be converted into water and carbon dioxide by means of a series of reactions commonly grouped together and called the *Krebs tri-carboxylic acid cycle*. Since citrate is a key tricarboxylic intermediate, the Krebs cycle is often synonymously referred to as the *citric acid cycle*. The use of the word cycle implies that the first step of the system initiates a process leading eventually to a product which participates as a reactant in the very same first step. Figure 86 outlines the reactions making up this cyclic system. Probably more is known about the Krebs cycle than any other phase of intermediary metabolism. Important synthetic mechanisms drawing upon the reactants of the cycle are shown to lead to the elaboration of alanine, aspartate, glutamate, and fatty acids. These diversions of raw material for growth from carbohydrate substrates will be considered subsequently.

A complex series of events introduces pyruvate into the Krebs cycle. The metabolic pathway for this process has been represented as follows:



Acetylcoenzyme A





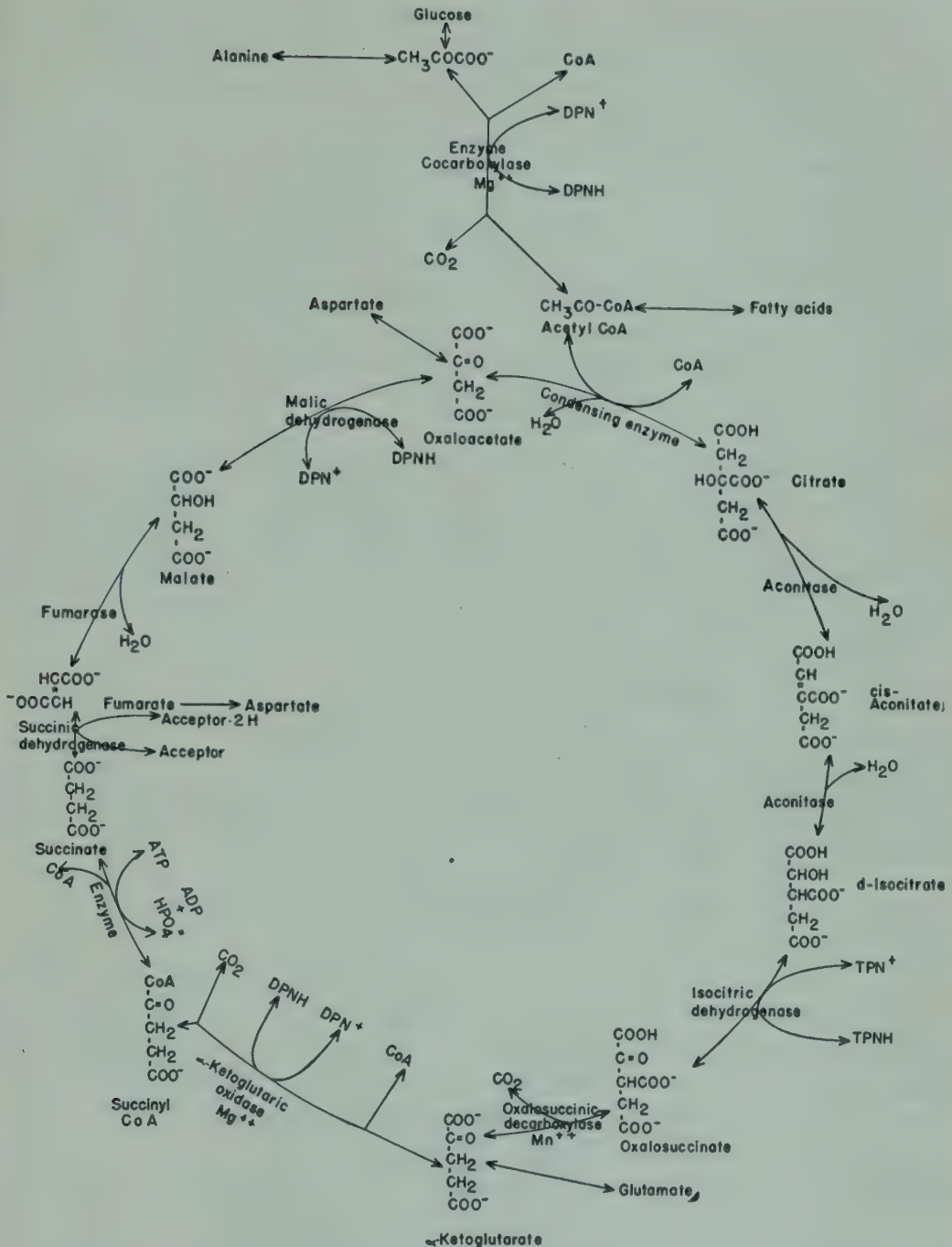
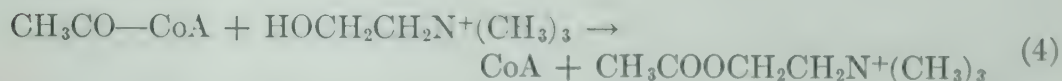


FIG. 86. The tricarboxylic acid cycle for the aerobic metabolism of pyruvate. The connections with other compounds than those derived from carbohydrates are indicated. It appears that all the reactions shown are reversible and may actually be reversed under certain conditions. However, the ordinary respiration of pyruvate proceeds in the clockwise direction.

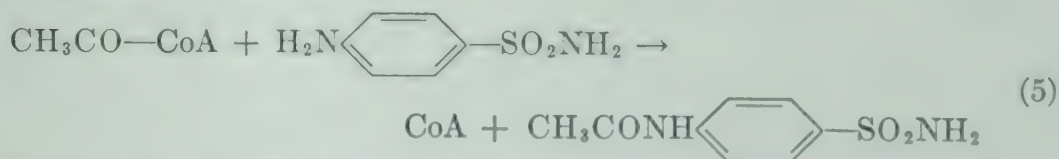
A large reproduction of this figure is in a pocket attached to the inside back cover. This has been provided for the convenience of the reader in following the material.

Reaction (1) is simply the addition of coenzyme A across the carbonyl bond of pyruvate and is thought to involve the thiol group of the coenzyme. Reaction (2) seems to be a typical oxidative decarboxylation requiring an enzyme,  $\text{DPN}^+$ , cocarboxylase, and magnesium ion. At this step one of the carbon atoms of the pyruvate is lost, two electrons are transferred to  $\text{DPN}^+$ , and the high energy compound acetylcoenzyme A results. In reaction (3) the activated methyl group of the acetylcoenzyme A condenses with oxaloacetate to produce citrate. The participating enzyme is called the condensing enzyme and has been isolated, crystallized, and rather thoroughly studied. Coenzyme A is restored to the system by reaction (3). The condensation might conceivably involve an addition across the carbonyl double bond of the oxaloacetate molecule and elimination of the coenzyme in the presence of water.

Acetylcoenzyme A is capable of transferring the acetyl group as shown in reaction (3) by means of condensations involving the methyl group. It also seems to react by splitting of the carbon-sulfur bond and the formation of ordinary acetyl derivatives. Thus various organisms including animals and *Lactobacillus plantarum* are able to form acetylcholine probably according to



Still other organisms utilize the high energy compound, acetylcoenzyme A, for the acetylation of various amines including the sulfanilamide drugs in detoxification processes.



A second type of acetylation reaction belonging to this class involves phosphate. Animals do not carry out this reaction but it seems to be common among bacteria, having been described for several species.



#### Acetylphosphate

Reaction (6) is reversible and converts one high energy compound into the other, thus permitting bacteria to utilize acetylphosphate in the condensation to citrate or any of the other acetylation reactions. Bacteria are thereby enabled to synthesize citrate from pyruvate, acetylphosphate, or acetate plus ATP since all of these compounds ultimately lead to the formation of acetylcoenzyme A.

Citrate and other tricarboxylic acids have been written in Figure 86 and various individual reactions as the doubly charged ions. In each case this is the predominant state of ionization at the pH of most natural systems, but the charges will actually be distributed statistically both in number per molecule and in their location on the molecule.

Aconitase catalyzes the conversion of citrate through *cis*-aconitate to isocitrate. The process involves elimination of water which switches the

hydroxyl group from its original position to an adjacent CH<sub>2</sub> group. In the equilibrium mixture produced by aconitase there is 89 per cent citrate, 3 per cent *cis*-aconitate, and 8 per cent *d*-isocitrate. The net result is surprising in one respect, namely, in the formation of an optically active substance from the optically inactive compound citrate. This problem is considered in this chapter in the separate section on optical isomerism in biology.

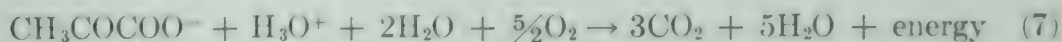
Isocitrate is dehydrogenated in the presence of TPN<sup>+</sup>, rather than DPN<sup>+</sup>, and decarboxylated to  $\alpha$ -ketoglutarate. The oxidizing activity is induced by isocitric dehydrogenase and the decarboxylation by Mn<sup>++</sup> and oxalosuccinic decarboxylase. The assumed intermediate is oxalosuccinate which may have but a fleeting existence, for the two enzymatic activities have not been separated so far. This complication has been interpreted tentatively on the basis that both reactions occur at the surface of a single enzyme. If this is so, oxalosuccinate would not have a truly independent existence but be attached to the enzyme. At any rate, in spite of the unknowns in the details of the process, this stage marks the second loss of a carbon atom as carbon dioxide in the Krebs cycle.

The  $\alpha$ -ketoglutarate undergoes oxidative decarboxylation in the presence of Mg<sup>++</sup>, DPN<sup>+</sup>, coenzyme A, and an associated enzyme system, and perhaps cocarboxylase. Reactions of this type obviously are complex and would be expected to proceed by a series of reactions. This process liberates the third atom of carbon as carbon dioxide, and from this point on the cycle merely serves to bring the level of oxidation up to that of oxaloacetate which is then again available for condensation with acetyl-CoA to repeat the cycle. The succinylcoenzyme A is a high energy compound and may conceivably serve for the transfer of succinyl groups to suitable acceptors. This particular possibility has not been explored, but the reaction written in Figure 86 is of importance both in the formation of succinate and in the transfer of inorganic phosphate to ADP.

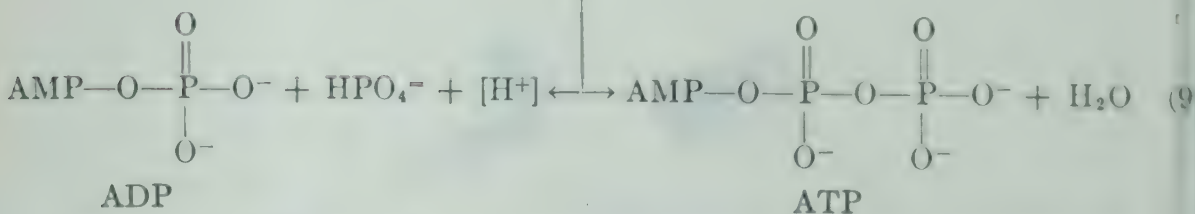
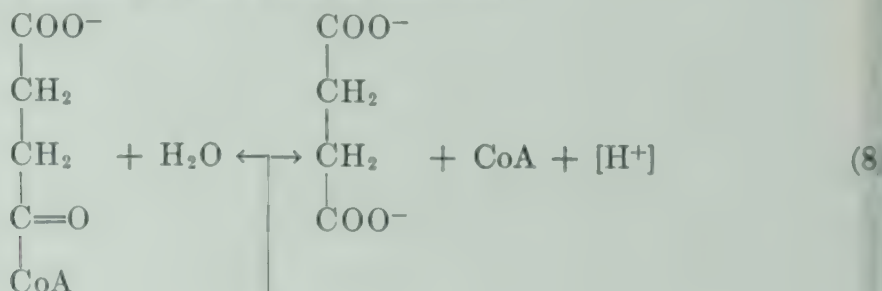
Succinate is dehydrogenated by succinic dehydrogenase, and eventually the pair of hydrogens so removed is transferred to oxygen and two molecules of ATP are generated. Succinic dehydrogenase has been obtained only in the form of particulate preparations which contain all of the components transferring the hydrogen to water. This difficulty has prevented



an understanding of the origin of the two high energy ATP molecules since the particulate material has resisted separation into individual enzyme preparations required for the experimental elaboration of the general mechanism. The fumarate formed subsequently reacts with water to form malate which is dehydrogenated in a typical way by DPN<sup>+</sup> to oxaloacetate, marking one complete cyclic operation and the overall oxidation of one molecule of pyruvate. The net result is



Thus in one clockwise turn of the cycle of Figure 86 starting with pyruvate, three molecules of carbon dioxide are formed at the steps indicated and represent the oxidation of the three carbon atoms of the pyruvate. According to reaction (7) as balanced, both hydrogen ion and water participate in this complete oxidation of pyruvate. The reactions of Figure 86 show that in the Krebs cycle water is both consumed during the formation of citrate, isocitrate, and malate and formed during the reaction yielding cis-aconitate. Furthermore, water is important in the formation of succinate although the figure as prepared does not show this since no net loss or gain of water occurs. In this particular step resulting in the formation of succinate, water is involved in a coupled system so that the events in the overall reaction happen simultaneously or very nearly so. However, in order to follow the water through this step, the reaction may be regarded for demonstration purposes only as consisting of two parallel reactions:

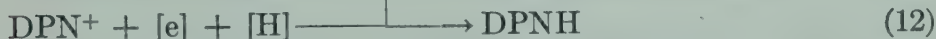
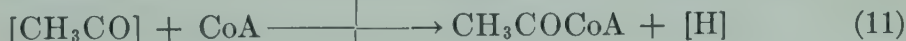
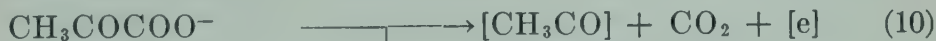


The symbol [H<sup>+</sup>] is used to represent the proton transferred from one step (8), to the other, (9). The proton probably reacts with water to form the hydrogen ion, H<sub>3</sub>O<sup>+</sup>, which functions as a proton carrier. The molecule of water in (8) hydrolyzes the succinyl coenzyme A to succinate (and the

proton) plus CoA. It will be observed that the second hydrogen atom from the water is acquired by the coenzyme A when the hydrolysis occurs. Reaction (8) thus consumes one molecule of water. The coupled reaction (9) then forms a molecule of water so that there is no net change in the amount of water. However, it will be clear that when ATP is subsequently utilized one water molecule is necessary. Therefore, if the complete metabolic picture is considered, a net quantity of one molecule of water is used as a result of the succinyl-CoA to succinate step since there is recycling of ATP in the natural situation. Hence, a net total of three molecules of water are consumed at each clockwise turn of the Krebs cycle and its associated reactions.

DPN<sup>+</sup> and TPN<sup>+</sup> are shown to be reduced at a total of four points in Figure 86. In each of three of these reactions (oxidation of isocitrate,  $\alpha$ -ketoglutarate, and malate) a proton is formed, though omitted from the figure. This particle reacts promptly with water to form a hydrogen ion. However, the DPNH and TPNH are subsequently oxidized, and at that time the proton is taken up again. Hence, there is no net change in the amounts of either water or protons in these three steps. More details on the oxidation of DPNH and TPNH are presented later.

When pyruvate is oxidized a proton is not formed as is shown by the partial reactions (10), (11), (12).



Reaction (13) is the sum of the others and represents the actual process since none of the quantities in brackets would have any appreciable existence. It is clear that a proton does not appear, yet one is actually consumed when the DPNH is oxidized in the usual way. Therefore, the overall result is the disappearance of one proton.

In reaction (7), representing the overall oxidation of pyruvate, the proton is written as combined with water and the requirements for the process thus become one hydrogen ion and two molecules of water.

Ultimately water is formed during the overall oxidation of pyruvate at the stage of oxygen utilization. One pair of hydrogen atoms (with electrons) are transferred through the succinic dehydrogenase system to an oxygen atom. Four pairs of hydrogens are transferred to oxygen through DPN<sup>+</sup> and TPN<sup>+</sup>. As a result five oxygen atoms are taken up and five molecules of water appear. It will be obvious that some of this metabolically derived water is consumed in the cycle, but reaction (7) shows water of both reac-



tion and formation in order to make it clear that five pairs of hydrogen atoms are acting in the electron transferring systems of respiration.

Throughout the foregoing discussion, the Krebs tricarboxylic acid cycle has been accepted as fundamental in the terminal respiration of pyruvate. In animals this assumption is justified and has been substantiated by a variety of critical experiments. Indeed, this citric acid cycle theory accounts for all of the pertinent data at hand on animal respiration. Concerning bacteria, however, a debate has been waged for some years as to the existence of such a pathway.

In attempting to demonstrate the citric acid cycle in bacteria difficulties are encountered. For a long time no one was able to prepare a cell-free system from bacteria capable of carrying out the steps of the Krebs cycle. A number of species are known to contain the necessary enzymes, but the mere presence of necessary enzymes does not provide definitive information regarding their order of action in a possible metabolic pathway. Furthermore, living bacteria are usually unable to metabolize added tricarboxylic acids. This negative finding was interpreted as evidence against the existence of the Krebs cycle in bacteria. It is known that dicarboxylic acids are metabolized and that these are involved in some sort of a cyclic process. Figure 87 shows the scheme of aerobic metabolism utilizing a dicarboxylic acid cycle which has had rather wide acceptance until very recently.

Of the reactions shown in Figure 87 those leading to acetate, fumarate, malate, and oxaloacetate are like those of the tricarboxylic acid cycle and would involve the same enzymes. The oxidative combination of two acetates to yield succinate is postulated on the basis of tracer data which were thought to be incompatible with the Krebs cycle. However, it appears that the data in question might also result from the operation of the latter cycle owing to the unsymmetrical behavior of citrate alluded to earlier and to a lack of equilibrium with added compounds. The decarboxylation of oxaloacetate can be induced by oxaloacetic decarboxylase which is widely distributed in bacteria.

Recent studies have shown that permeability may be the factor preventing living bacteria from metabolizing added tricarboxylic acids. It is now known that killed *Escherichia coli* preparations are able to metabolize citrate just as normal animal cells appear to do. Furthermore, cell-free extracts of *Azotobacter vinelandii* have been obtained which oxidize acetate rapidly in the presence of a catalytic amount of any one of the compounds in the citric acid cycle. This effect is known as the *sparking reaction*. All of the members of the cycle except possibly succinate are oxidized rapidly by the extracts although the intact bacilli were unable to oxidize added tricarboxylic acids and could oxidize the dicarboxylic acids only after a lag period. In a similar way resting cell suspensions of *Pseudomonas aeruginosa*



oxidize a number of the intermediates of the Krebs tricarboxylic acid cycle only after a period of adaptation. However, dried cells with altered permeability properties oxidize all of the intermediates immediately. Therefore, it seems that the permeability properties of bacteria discussed in an earlier chapter are of singular importance in the problem of establishing

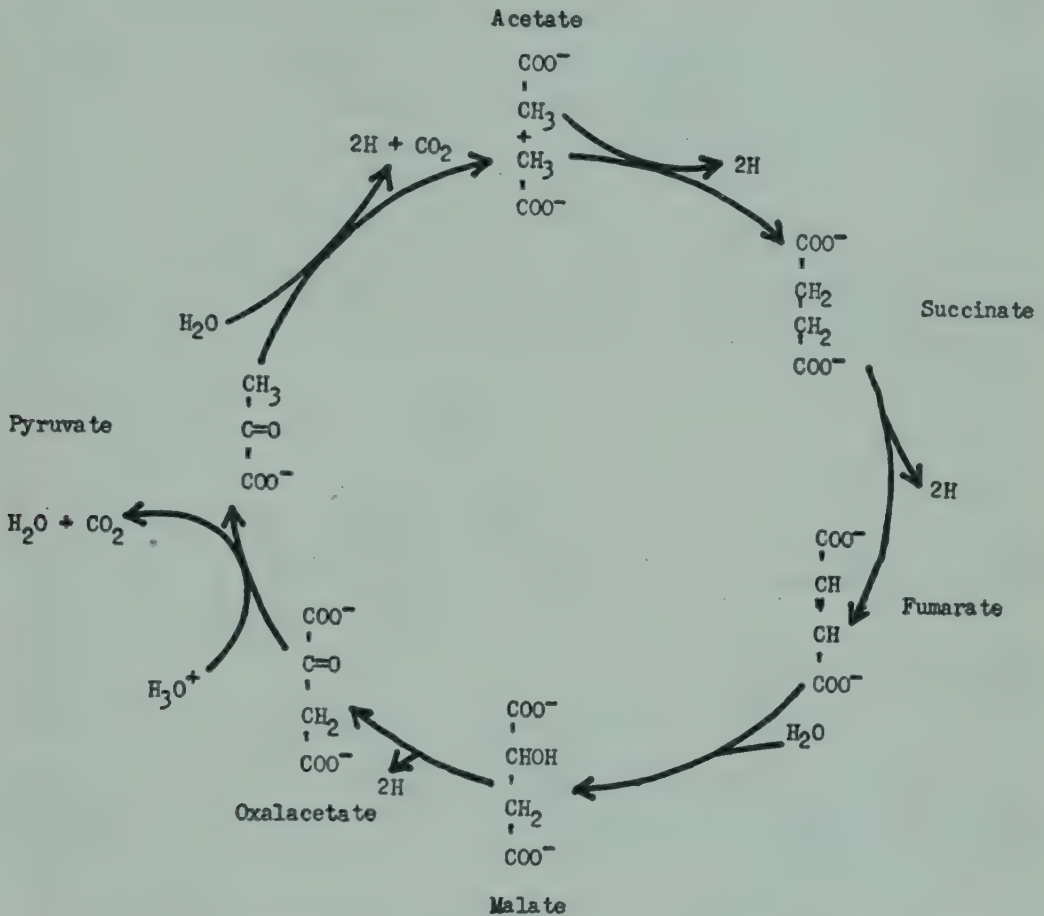


FIG. 87. The dicarboxylic acid cycle postulated for the respiration of bacteria. Steps involving electron transfers are shown conventionally as yielding  $2\text{H}$  for simplicity rather than the reduced coenzymes which actually are found.

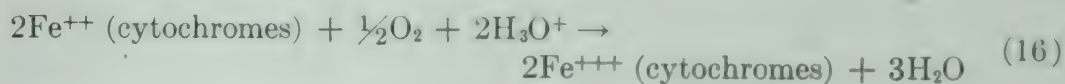
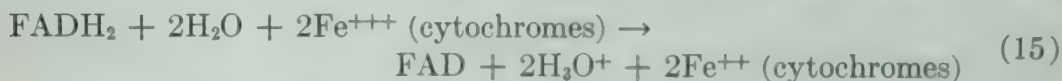
the existence of the citric acid cycle. Lastly, the consensus of the most recent reports indicates a growing conviction that the Krebs tricarboxylic acid cycle is the basic terminal aerobic mechanism in the oxidation of glucose, pyruvate, and acetate by many bacteria.

The chemical free energy of pyruvate does not appear in the Krebs cycle as useful high energy phosphorus compounds except in the one step of the conversion of succinylcoenzyme A to succinate. This reaction generates one ATP from ADP per each pyruvate molecule and represents only

a small fraction of the total free energy change occurring when pyruvate is completely oxidized. Since in fact much of the remaining energy does become available to the organism, the question is raised of how this comes about. Aerobic metabolism apparently provides most of the useful free energy by means of reactions coupled to the tricarboxylic acid cycle rather than by reactions directly in the cycle itself.

One reaction of importance is that of the dehydrogenation of succinate to fumarate. As already mentioned the two hydrogen atoms removed from the succinate are transferred by an unknown mechanism to an oxygen atom. Somewhere in this reaction sequence two molecules of ATP are formed for each succinate ion oxidized. The other significant reaction systems in the utilization of free energy involve the stepwise oxidation of DPNH on the one hand and TPNH on the other. Most of the energy obtained from respiration comes from these oxidations and a good deal of it is stored in ATP.

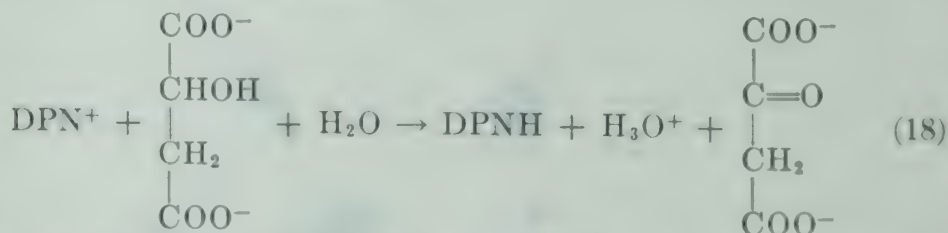
The known oxidative steps starting with DPNH may be represented as follows:



The sum of these reactions is



The hydrogen ion consumed in the above reaction is formed during the reduction of  $\text{DPN}^+$  in reactions in the cycle as for example in the reaction mediated by malic dehydrogenase:



Hence  $\text{H}_3\text{O}^+$  is formed by reaction (18) and immediately disappears by way of reaction (14). In reactions (14) and (15) FAD represents flavineadenine-dinucleotide usually present as a prosthetic group on a protein. The combination is known as a flavoprotein and there are several different such substances differing in the nature of the protein moiety and consequently in their oxidizing power. FAD is composed of riboflavin, two phosphates,

ribose, and adenine arranged in the order mentioned, and when reduced two hydrogen atoms per molecule are moved onto the riboflavin portion. Certain flavin systems appear to contain only the riboflavin phosphate giving them different oxidation characteristics than the dinucleotide types of flavins.

The iron-porphyrin-proteins known as the cytochromes are reduced in the presence of cytochrome reductases, and subsequently the last member of the series is oxidized by oxygen in the presence of cytochrome oxidase. There are three known cytochromes, *a*, *b*, and *c*, in the electron carrying chain of coenzymes of animals. The three cytochromes transfer electrons from one to the next.

It is customary to express the tendency of materials to accept electrons by means of the oxidation-reduction potential under a standardized set of conditions. These values are collected in Table 49 for the compounds transferring electrons to oxygen. From the differences between adjacent stages one may calculate the free energy changes corresponding to these electron changes by means of the equation

$$\Delta F^{\circ} = nF_e$$

where the free energy change = (2) (23,068) (voltage difference). In the table the electrons are transferred from DPNH upward in order through the compounds listed to oxygen as the terminal acceptor.

It will be recalled that the free energy change on the hydrolysis of one of the phosphates of ATP is about 10,500 cal. Thus one observes free energy changes of similar or greater magnitudes at three steps and a search for the appearance of ATP might be made at these points. Indeed the jump from cytochrome *a* to oxygen is large enough to provide two molecules of ATP of a total of four possible, assuming that 9200 is not significantly different from 10,500 in view of the uncertainties as to the exact value of both quantities. The two small changes contribute energy to the metabolic system and this might be useful as heat in driving some of the slightly endergonic reactions.

Present evidence suggests that the electron transfer from DPNH to oxygen supplies three high energy phosphates. Therefore, all of the energy available at suitable levels may not be actually converted into high energy phosphates. Perhaps a mechanism is not available at one step, or perhaps one stage is complex consisting of several reactions which break the energy transfers into steps too small to provide energy for the phosphorylation of ADP. Several investigators have claimed that phosphorylations do not occur once electrons have passed into the cytochrome system. The data used to support this conclusion have been challenged. It would seem from Table 49 that only one phosphorylation could occur between DPNH and



cytochrome *b*. Therefore, other phosphorylations, if they occur, must take place at the subsequent stages of the electron transfer in the cytochrome system.

Intensive research is being devoted to the problems of terminal respiration, and important observations in this fundamental phase of metabolism may be expected. For example, different bacteria produce different cytochromes which will have different redox potentials. At least nine bacterial cytochromes exist, probably never all in any one organism. In any case it is clear that the electron transfer system of the sort shown in Table 49 will probably differ somewhat from species to species.

Another fundamental problem is that of the actual mechanism of forma-

TABLE 49  
*Redox potentials of and free energy changes for the interaction of known members of the system transferring two electrons stepwise from bottom to top at pH 7 and 30°C*

	REDOX POTENTIAL IN VOLTS	POTENTIAL DIFFERENCE	$\Delta F^\circ$ PER PAIR OF ELECTRONS
Oxygen.....	+0.80		
Cytochrome <i>a</i> .....	+0.29	0.51	-24,000
Cytochrome <i>c</i> .....	+0.27	0.02	- 900
Cytochrome <i>b</i> .....	-0.04	0.31	-14,300
Flavoprotein.....	-0.08	0.04	- 2,000
Diphosphopyridine nucleotide (reduced) .....	-0.28	0.20	- 9,200
Hydrogen.....	-0.42		
Transfer of electron pair from hydrogen to oxygen.....		1.22	-56,000

Adapted from Ball (1944).

tion of high energy phosphorus compounds during electron transfer from DPNH to oxygen. It has been proposed that some of the metabolic intermediates may participate and more specifically that a dicarboxylic acid cycle might be involved in the first step, the oxidation of DPNH by FAD. Figure 88 outlines this hypothesis. The important features of this hypothetical formulation include the connection to the tricarboxylic acid cycle, oxidation of DPNH, reduction of FAD, recovery of the oxaloacetate, and formation of ATP. The cycle probably could not be reversible to any appreciable extent because of the enolization phase whose equilibrium would be far in the direction of the keto form of oxaloacetate. Mechanisms for phosphorylations coupled with electron transfers in the cytochrome system have not been advanced so far since most of the attention has been devoted to the primary problem of demonstrating the presence or absence of such phosphorylations.

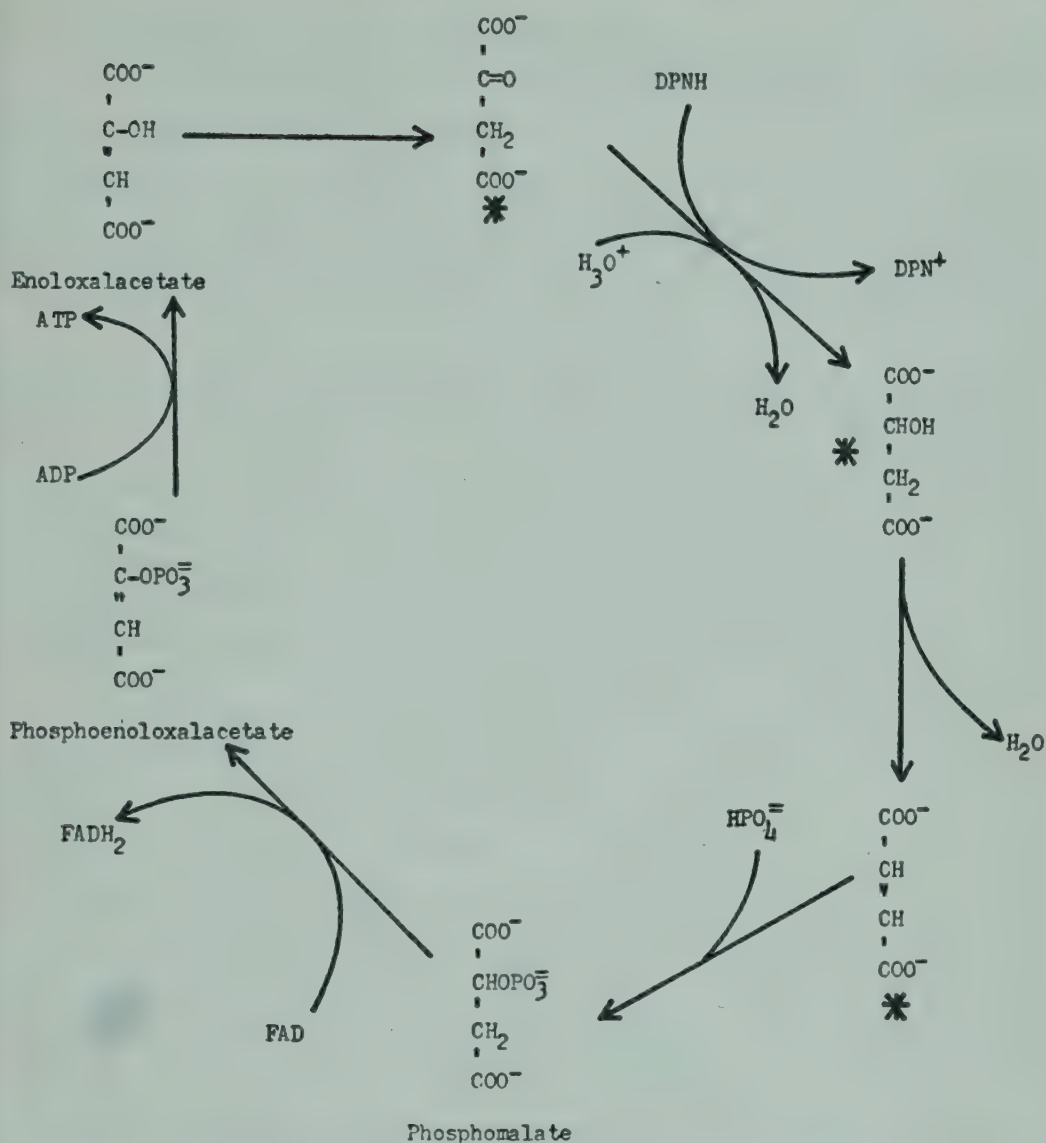
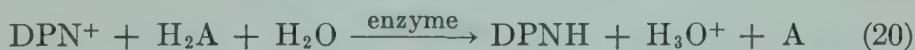


FIG. 88. A speculative scheme for the formation of ATP when electrons are transferred from DPNH to FAD. Note the three points (marked by asterisks) in common with the Krebs cycle to which this system would be attached.

When DPN<sup>+</sup> is reduced as in reaction (18), a hydrogen ion appears as shown by the general process



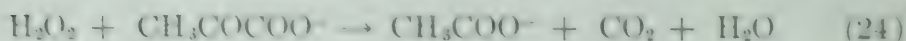
where H<sub>2</sub>A is a hydrogen donor and A is the oxidized form of that substance. Since TPN<sup>+</sup> works in the same way four hydrogen ions appear for each pyruvate oxidized by the tricarboxylic acid cycle. If the cycle existed in an isolated environment a profound pH change would occur. However, the

cycle is not isolated in the cytoplasm and the hydrogen ions are removed immediately by reduction of flavins.



In the hypothetical scheme of Figure 88 the hydrogen ion is consumed in the oxaloacetate to malate step. Therefore, it seems that the  $\text{H}_3\text{O}^+$  acts in a catalytic manner, being formed when  $\text{DPN}^+$  is reduced and consumed when  $\text{DPNH}$  is oxidized. Water itself behaves the same way as far as reactions (20) and (21) are concerned.

There are some respiratory reactions in which flavoproteins appear to transfer hydrogen directly from a particular substrate to oxygen without mediation by the cytochrome system. Various types of compounds may be oxidized in this way, and the necessary oxidases have been observed in several bacterial species. Instead of water, hydrogen peroxide is the primary product, and this toxic product is usually converted by enzymatic action to water and oxygen. When catalase is absent some other reaction may dispose of the peroxide. *Mycobacterium phlei* contains an enzyme system illustrating these mechanisms, one that carries out the reactions:



In reactions (22) and (23) the FAD and  $\text{FADH}_2$  are actually the prosthetic groups of a flavoprotein-enzyme which is involved in both reactions. Removal of peroxide (24) is a rapid and spontaneous step. Catalase is practically absent. Although a number of similar systems are known, the possibility of their coupling to a phosphorylation mechanism has not been reported.

A comparison of the useful energy made available by fermentation and respiration is presented in Table 50. It is apparent at once that high energy phosphate is obtained in much greater yield from the aerobic than from the anaerobic dissimilation of a given quantity of glucose. Therefore, one would expect greater growth of facultative organisms under conditions of respiration than during fermentation. If a limited quantity of substrate is available, the organism can accomplish more work when using oxygen as the final hydrogen acceptor. Even when the substrate is unlimited in quantity aerobic growth might be more rapid than anaerobic growth because the rate of production of high energy compounds could be reduced by the sheer bulk of substrate that must be handled during fermentation. In this situation the term growth is used in the strict sense, since both multiplication and the assimilation of cellular material without accompanying cell division are affected.



Table 50 is based on the accepted classical pathways of fermentation and respiration. Involvement of other systems could either decrease or increase the possible energy yields. If high energy substrates are used, many organisms are able to conserve this energy as shown earlier and reduce the requirement for ATP by eliminating (at least partially) the need for this compound in the glucose to glucose-6-phosphate step. On the other hand, fermentation by the pentose pathway appears to yield one less high energy

TABLE 50

*Probable yields of high energy phosphorus compounds in fermentation and respiration*

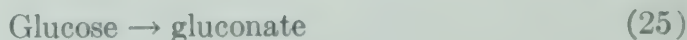
REACTION	PRIMARY ELECTRON ACCEPTOR	PROBABLE YIELD	
		Fermentation	Respiration
3-Phosphoglyceraldehyde → 1,3-diphosphoglycerate.....	DPN <sup>+</sup>	0	3
1,3-Diphosphoglycerate → 3-phosphoglycerate....	—	1	1
Phosphoenolpyruvate → pyruvate.....	—	1	1
Pyruvate → acetylcoenzyme A.....	DPN <sup>+</sup>		3
Isocitrate → oxalosuccinate.....	TPN <sup>+</sup>		3
α-Ketoglutarate → succinylcoenzyme A.....	DPN <sup>+</sup>		3
Succinylcoenzyme A → succinate.....	—		1
Succinate → fumarate.....	?		2
Malate → oxalacetate.....	DPN <sup>+</sup>		3
Total per glucose metabolized.....		4	40
High energy phosphate consumed in			
Glucose → glucose-6-phosphate.....		1	1
Fructose-6-phosphate → fructose-1,6-diphosphate.....		1	1
Net gain per glucose molecule.....		2	38

phosphate than does the Embden-Meyerhof pathway and thus proportionally would represent a very high reduction in available energy. Respiration through the pentose route and the citric acid cycle should produce relatively almost as much ATP as by the Embden-Meyerhof and citric acid cycle.

The aerobic metabolism of glucose, as has been suggested, may occur by the pentose pathway. In fact, it has been proposed that the major portion of respiration of some animal tissues occurs by this mechanism, and the process has been reported for yeast. The hypothesis has been advanced that a pentose derivative is formed as was shown in Figure 84 but that this compound is further degraded one carbon at a time to the triose stage

instead of undergoing a primary split into both two and three carbon fragments. Either of these schemes may describe the metabolism of those species of bacteria which contain no aldolase and consequently are unable to utilize the Embden-Meyerhof pathway.

Still another aerobic mechanism of dissimilation appears to operate in *Pseudomonas aeruginosa*, an obligate aerobe. This organism carries out the first steps of glucose degradation without phosphorylations. The known reactions are



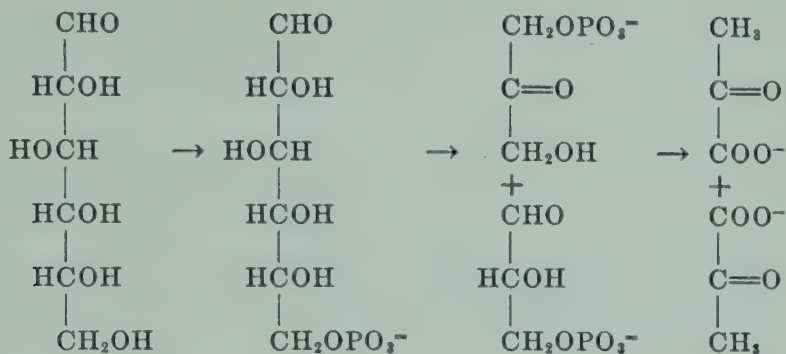
This mechanism is the first one described which definitely indicates non-phosphorylating oxidations at the initial stages of carbohydrate metabolism. Phosphorylated hexoses are not formed although phosphorus compounds do appear subsequently. The energy recovery from reactions (25) and (26) is not understood but may involve high energy phosphates generated further down in the electron transferring systems.

The discussion of the Krebs citric acid cycle centering about Figure 86 was based upon a combination of "active" acetate with oxaloacetate to yield citrate. The data supporting this mechanism seem valid, yet as mentioned the scheme may not be universal. Some bacterial species may accomplish a combination of four and three carbon intermediates resulting in a seven carbon product thought to be a member of the respiratory cycle. Data indicative of such reactions have been published, and the isolation of a seven carbon tricarboxylic acid has been claimed.

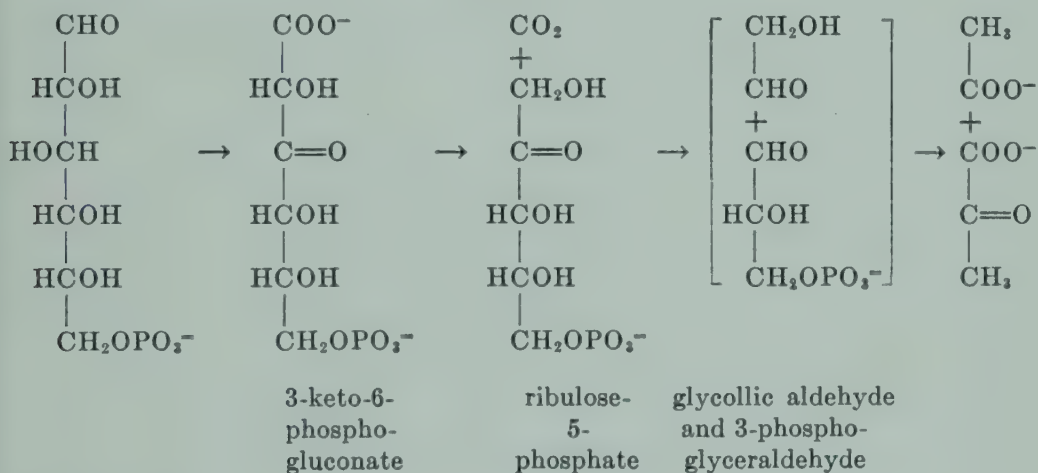
Although all of the details are not available for the various pathways of carbohydrate metabolism, it is becoming increasingly clear that different species may use different systems. This notion is somewhat of a departure from the older opinion of comparative biochemistry which postulated but a single major mechanism for the intermediate metabolism of carbohydrates.

Figure 89 outlines the steps in the three most probable schemes for the initial steps in glucose dissimilation. The carbon atoms in the figure follow horizontal paths. In other words, the positions of carbon atoms in the various products are written in the same order as the positions in the glucose molecule from which they are derived. In the Warburg-Dickens-Lipmann-Horecker scheme the existence of 6-phospho-3-ketogluconate is postulated but has not been demonstrated. The glycollic aldehyde and 3-phosphoglyceraldehyde are postulated as intermediates but other compounds are possible in both cases. It is actually even probable that the two carbon fragment is some substance other than glycollic aldehyde. The Campbell pathway leads to pyruvate but the fate of the individual carbons of glucose

## Embden-Meyerhof



## Warburg-Dickens-Lipmann-Horecker



## Campbell

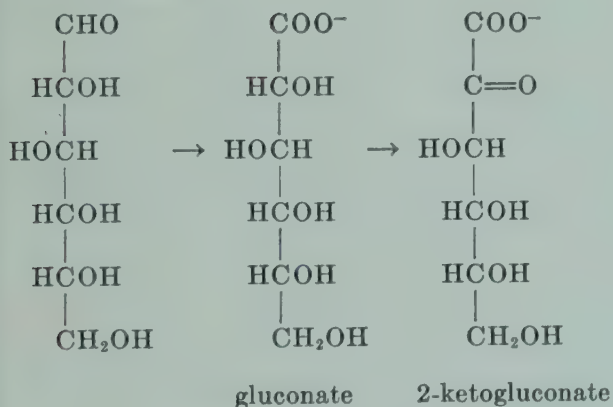


FIG. 89. The fate of the carbon atoms of glucose by three pathways of intermediate metabolism. Open chain formulas are used to permit an easier comparison of the positions of given carbon atoms as the processes proceed.



has not been traced. It is difficult to be sure which mechanism is the most widespread and how many organisms may actually possess more than one pathway. Probably all three of the systems of Figure 89 are important, and the last mentioned may be the common mechanism among the microorganisms that are obligate aerobes.

**THE PASTEUR EFFECT.** Muscle tissue and facultative organisms are provided with metabolic pathways that function in either the presence or absence of oxygen. It has been repeatedly shown that facultative species capable of utilizing molecular oxygen as the hydrogen acceptor grow with a lower consumption of carbohydrate per unit of new cellular material when oxygen is available and metabolism is aerobic. The economics of aerobic and anaerobic metabolism have been compared elsewhere, so that here we wish to turn our attention to another phase of the phenomenon of two coexisting metabolic systems. In the field of metabolism current terminology includes *respiration* and *glycolysis* as terms which are applied respectively to the aerobic and anaerobic dissimilation of carbohydrates. This usage of the term *glycolysis* differs from that employed in older work on animal physiology which restricted the word to the breakdown of glycogen without reference to the presence or absence of oxygen. In the present instance in accordance with the literature of intermediate metabolism the term *glycolysis* will be applied to anaerobic systems and generalized to include the anaerobic dissimilation of carbohydrates other than glycogen.

While respiration clearly cannot occur at least in its later stages without oxygen, there is no *a priori* reason to assume that glycolysis ceases when oxygen is available, since facultative organisms may still have the enzymatic means for fermentation. Yet most organisms do not carry out glycolysis while respiration is taking place. This somewhat unexpected inhibition of glycolysis during aerobic metabolism has been given the name *Pasteur effect* in honor of Pasteur who first observed the phenomenon.

Two general causes for the Pasteur effect may be postulated. Either respiration produces a substance that inhibits glycolysis, or respiration consumes an intermediate essential for glycolytic processes. Various specific applications of both of these possibilities have been advanced and supported experimentally. The problem has been a difficult one to settle, however, and in spite of extensive work a conclusive answer has not been reached. Several theories have been proposed and some of these have merit although they would appear to be mutually exclusive. It may be, of course, that the Pasteur effect is the resultant of a variety of influences and is variable in its cause which depends upon the nature of the species.

Among the hypotheses now regarded as the most promising are those postulating the inhibition of various enzymes. Evidence suggests that an iron enzyme may be involved in glycolysis. It is thought that this enzyme is

catalytically active only when the iron is in the ferrous state and that oxygen inactivates the enzyme by oxidation of the iron. Another concept is based upon the presumed oxidation of —SH groups in particular enzymes thus preventing one or more of the glycolytic reactions. It is not yet possible, however, to localize the postulated oxidation in such a way as to avoid inactivation of all the other necessary iron or —SH enzymes in the organism.

Perhaps the most satisfactory explanation of all is one concerning the role of phosphate. It appears that a higher concentration of phosphate is necessary for glycolysis than for respiration. Since respiration results in the availability of much higher energies in the cell and consequently in a greater conversion of phosphate to ATP, the phosphate concentration may become too low for glucose utilization by a fermentative pathway when aerobic respiration is proceeding rapidly. In other words, oxygen may favor processes that block glycolysis by keeping phosphate largely in bound forms. If so, then the addition of special enzymes capable of splitting the high energy phosphates should allow both glycolysis and respiration to proceed simultaneously. This effect has been observed.

Several compounds are known to inhibit the Pasteur effect and to permit both glycolysis and respiration in the presence of oxygen. With some of these agents it has been shown that phosphorylation reactions are dissociated from respiration, thus indicating that the Pasteur effect does indeed depend upon control of the phosphate concentration. If all of these various inhibitors can be shown to act in the same way, the existence and nature of a general mechanism of the Pasteur effect will finally have been settled.

On the other hand, phenomena that complicate the picture have been observed with certain molds. These particular organisms dissimilate carbohydrate by an apparently typical process yielding lactate by reduction of pyruvate. If oxygen is admitted to the system this glycolysis continues and may even increase, contrary to the usual manifestation of the Pasteur effect. Indeed, it is assumed that the pyruvate competes successfully with oxygen for the available hydrogen donors. Be that as it may the additional problem of the lack of a Pasteur effect in these molds now needs an explanation.

#### METABOLISM OF NITROGEN COMPOUNDS

Inasmuch as nitrogen is a necessary component of many of the essential chemical compounds making up organisms, the reactions that compounds of nitrogen undergo in living systems are extremely important subjects for biochemical study. Although, as expected, the overall problem of the intermediary nitrogen metabolism is complex, promising beginnings have been made.



During the discussion of the metabolism of carbohydrates much emphasis was placed on the utilization of carbohydrates for energy. While it will become apparent that numerous nitrogenous materials may serve as sources of energy, the major emphasis will be directed toward reactions of synthetic value. The compounds of nitrogen are of more interest in their roles as enzymes, coenzymes, nucleic acids and amino acids than as potential sources of energy. Indeed, the utilization of such substances for their energy usually involves elimination of the nitrogen and conversion to organic intermediates from which energy is derived by the reactions already mentioned.

### *Ammonia*

Many species of bacteria are able to satisfy completely their requirements for nitrogen by utilizing ammonia (more exactly ammonium ions). Ammonium salts will provide nitrogen for the growth of *Staphylococcus aureus* although four strains in a series of 25 required tryptophane as an accompanying nutrient during the assimilation of the ammonia. This peculiar requirement has been found for several other species although it need not be immutable. Exacting strains of *Salmonella typhosa* require such small amounts of tryptophane that the amino acid cannot supply an appreciable amount of the total cellular nitrogen but rather must be functioning in a specific manner. Furthermore, these strains may be trained to utilize ammonium chloride as the sole source of nitrogen by growing them on media containing progressively decreasing amounts of tryptophane.

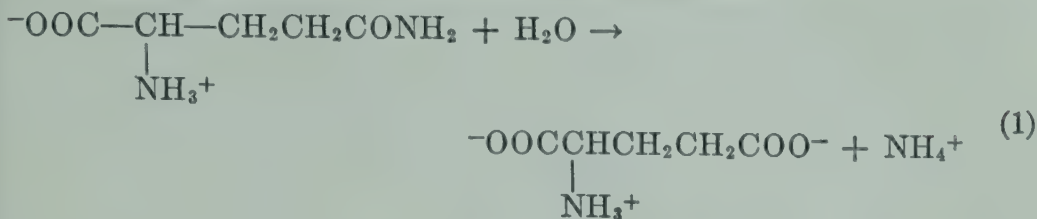
Resting cells of *Serratia marcescens* take up ammonium ions with a concomitant increase in the rate of respiration. The elevated level of respiration is maintained until the ammonium has been consumed and then falls to its original value. An analysis of the organisms reveals that the ammonium nitrogen is quantitatively incorporated into cellular material. Hence it seems that respiratory activity is associated with the supply of energy required for the assimilation of the ammonia.

After an exposure of three minutes to ammonium sulfate containing isotopic nitrogen *Azotobacter vinelandii* is found to have incorporated nitrogen into the cellular amino acids. Glutamic acid contained the highest proportion of the isotope, suggesting that this amino acid lies near the beginning of the series of reactions involved in the uptake of ammonium ion. Further details are presented in connection with the synthesis of amino acids.

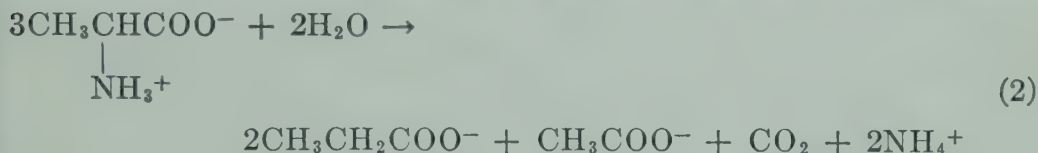
In addition to the anabolic role of ammonium ion, this ion may also appear as the product of metabolic reactions. For example, *Streptococcus pyogenes* intracellularly decomposes glutamine yielding ammonium ion



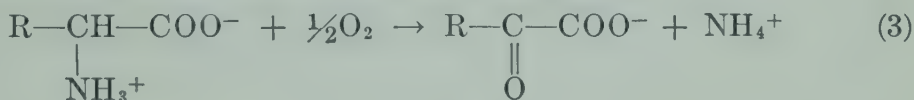
which then becomes available for synthetic purposes:



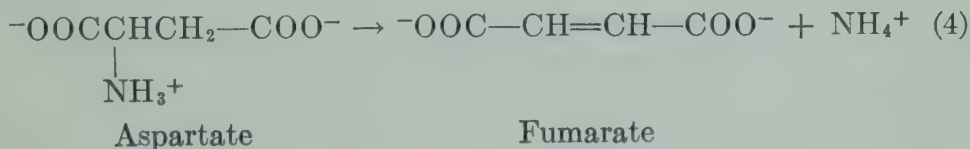
Reaction (1) is undoubtedly an overall process requiring enzymatic assistance for one or more steps. Other species are able to split the amino group off certain amino acids. In this connection, *Clostridium propionicum* ferments alanine yielding ammonia as a product by the following means,



and simultaneously gains from the process the energy needed for growth. On the other hand, deaminations that do not involve the appearance of energy in a useful form are quite widespread. In fact, there are strains of *Proteus vulgaris* which can oxidatively deaminate the natural isomers of 19 amino acids. The general reaction may be written



Anaerobic deaminations also are common, *Escherichia coli* actually acting more rapidly on serine and aspartic acid by the anaerobic mechanism than it does aerobically on glycine, alanine, and glutamic acid. The reaction involved appears to be:



### Amino Acids

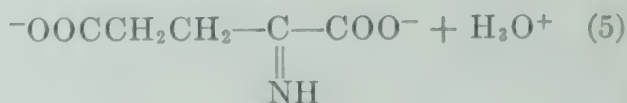
The metabolism of amino acids includes those reactions leading to the synthesis of peptides and proteins and also the hydrolyses of these materials into their constituent amino acids. However, the discussion of these processes is deferred to the section covering the metabolism of proteins. The other metabolic reactions in which amino acids participate serve either for the synthesis or the decomposition of the amino acids themselves. Insofar

as any reaction is reversible under biological conditions the reaction theoretically may serve either purpose. Let us consider first those processes which are considered to be important primarily in syntheses of amino acids.

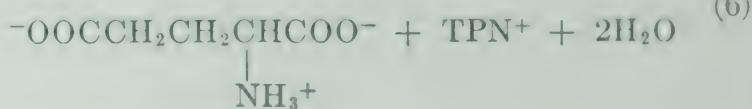
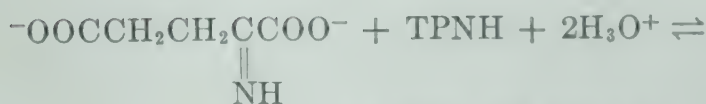
Evidence already alluded to indicates that glutamic acid is a key substance in the synthesis of amino acids. Furthermore, as has been shown, glutamate is connected to the Krebs tricarboxylic acid cycle at the  $\alpha$ -ketoglutarate stage. The reaction involves two known steps and is regarded as an important system for the incorporation of nitrogen into organic compounds of value to the organism.



$\alpha$ -ketoglutarate



$\alpha$ -iminoglutarate



glutamate

Reaction (5) appears to be spontaneous and reversible while reaction (6) is catalyzed by glutamic dehydrogenase and requires the reduced coenzyme. Since the overall system is reversible, any removal of glutamate favors further synthesis.

In addition to the glutamic dehydrogenase, *Escherichia coli* contains another enzyme, aspartase, that is capable of utilizing ammonium ions for the synthesis of amino acids. Aspartase converts fumarate to aspartate reversibly according to

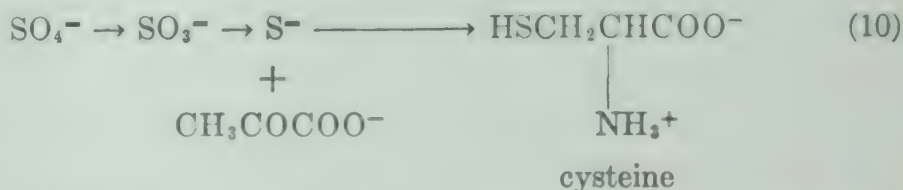


Many, if not all, bacteria are able to carry out various deaminations of other amino acids, but it is not known whether these reactions are readily reversible nor whether they are of any importance in the economies of a bacterium.

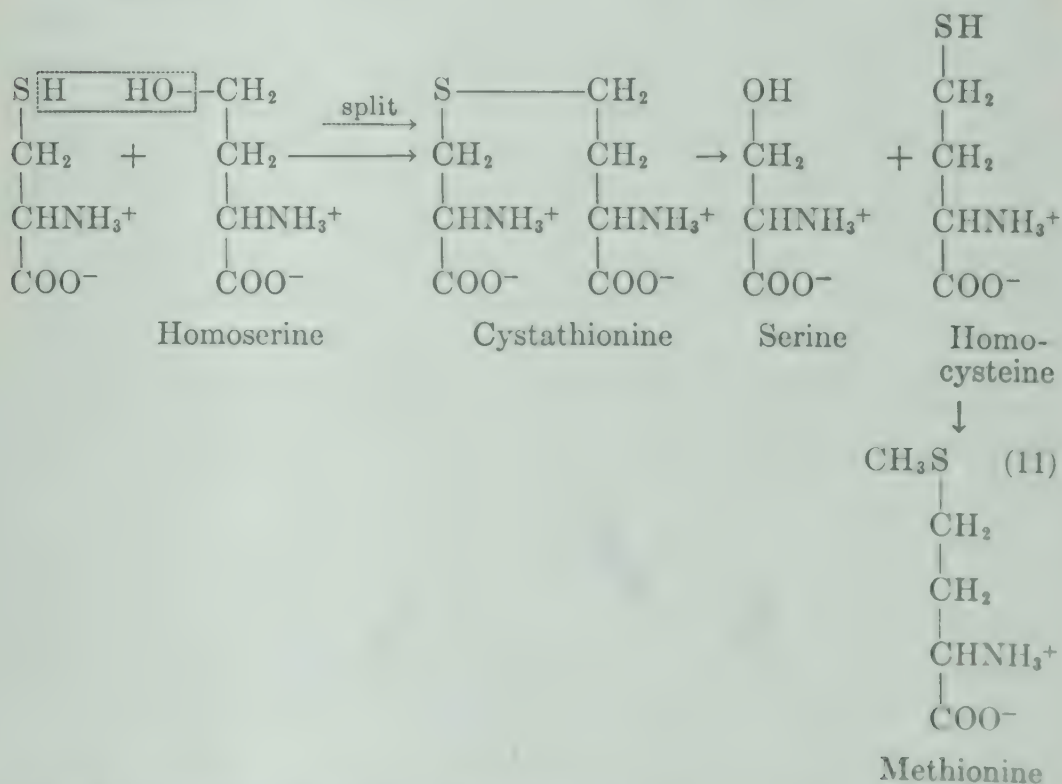




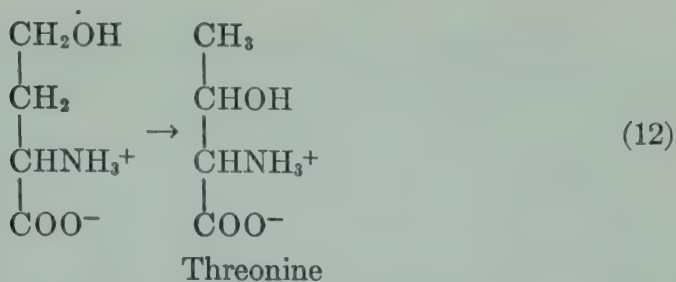
The use of biochemical mutants has also been employed very effectively in studies of the synthesis of cysteine and methionine. *Escherichia coli* is able to utilize sulfate as a source of sulfur for growth, synthesizing cysteine according to



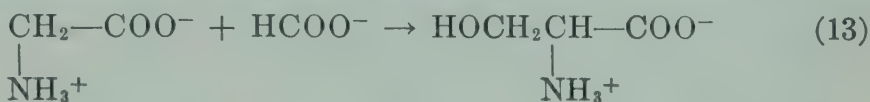
where the last step is obviously complex and has not been completely outlined. The cysteine is converted by *Escherichia coli* to methionine, but it has not been possible to discover the individual reactions in this conversion with mutants of the organism. However, studies with *Neurospora* reveal three reactions



The last step, the methylation of homocysteine, appears to require vitamin B<sub>12</sub> as a cofactor, and choline probably serves as the source of the methyl group. In addition to its participation in (11), homoserine is now known to be a precursor for threonine although the details have not been elucidated.

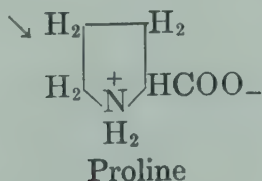
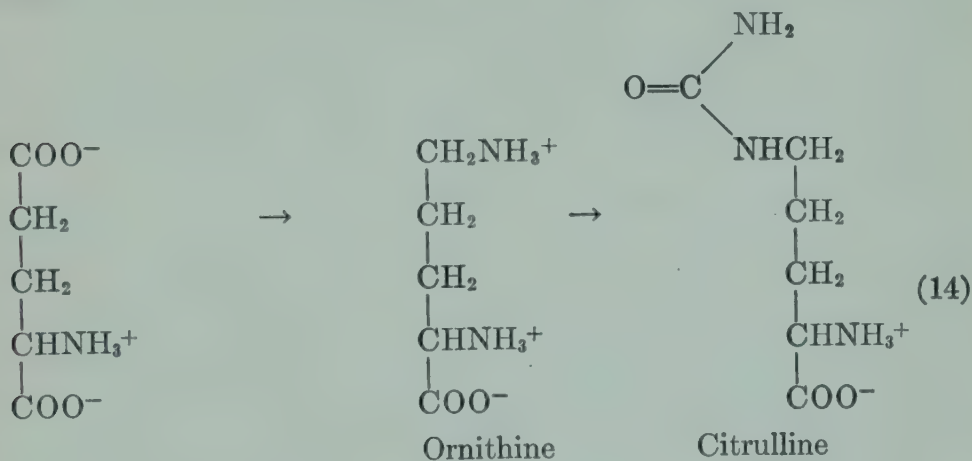


Serine, a key intermediate in the synthesis of tryptophane, is formed by *Streptococcus faecalis* from glycine and formate:

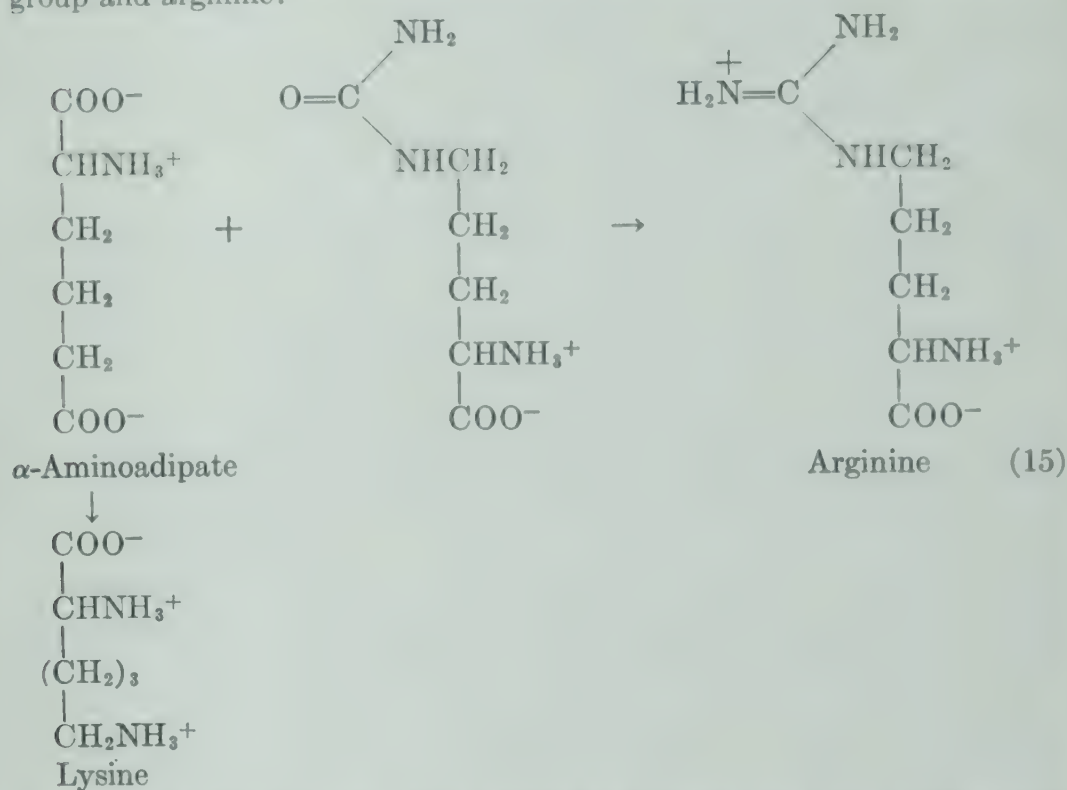


Cofactors are required in the process, pyridoxal and folic acid being effective in this organism. Partial reduction of the formate or of some condensed product of formate would seem to be necessary during the individual participating reactions. *Leuconostoc mesenteroides* is able to utilize carbon dioxide, glycine, pyridoxal and *p*-aminobenzoic acid for growth in the absence of serine. Synthesis of serine apparently occurs using the carbon dioxide as the source of the one carbon residue to be added to the glycine molecule and may represent a type of reaction for the fixation of carbon dioxide.

The central position occupied by glutamic acid is nowhere more evident than in the postulated mechanisms for the origins of proline, arginine and ornithine:



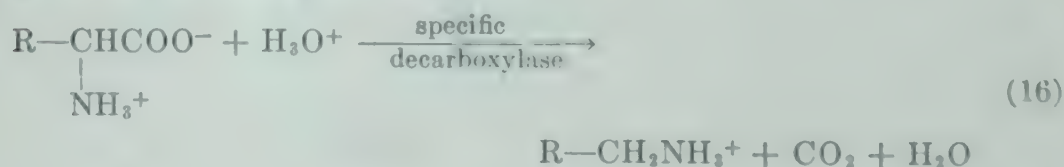
A nitrogen then seems to be transferred to the citrulline forming the guanido group and arginine:



While it is obvious that many details are missing and that the relationships presented may not be valid for all organisms, a start has been made in understanding the *in vivo* syntheses of amino acids. It is equally obvious that much further progress must be made in this direction before we become very well informed about the overall metabolism of bacteria and other forms of life.

The degradation of amino acids as carried out by bacteria involves several reactions which have already been presented. If a given amino acid is converted into another amino acid one considers the first as having been lost to the organism. However, since such events have been considered as synthetic processes, the reader is referred to that section. Likewise those reactions (2-4) yielding ammonium ions from amino acids have been outlined and would clearly be classified as degradations.

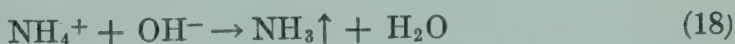
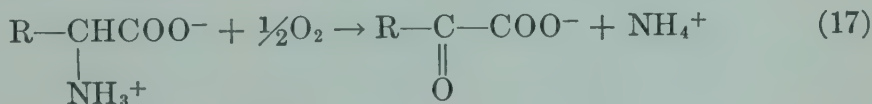
Instead of the amino group, bacteria may attack the carboxyl groups of amino acids. The general reaction is





and requires a specific *decarboxylase* for each amino acid as well as the coenzyme pyridoxal phosphate. Such reactions have been observed for a number of amino acids including lysine, arginine, ornithine, histidine, tyrosine, aspartic acid, glutamic acid, and phenylalanine. It should be noted that all of these amino acids except the last possess functional polar groups in addition to the usual single amino and carboxyl groups.

Various species of bacteria bring about different overall combinations of decarboxylation, deamination, hydrolysis, and oxidation depending upon the organism and the environmental conditions. The net effect observed may actually be the result of several consecutive reactions, only the first step being the degradation of an amino acid and the others being typical metabolic reactions of the materials formed from the amino acid. Therefore, one cannot actually describe these various overall processes as degradations of amino acids involving any first steps other than deamination or decarboxylation. In addition, no one has shown that both the deamination and decarboxylation of amino acids can occur in a given culture at one time. The enzymes responsible for these two processes are adaptive and are formed only under particular conditions of pH. For example, *Escherichia coli* produces glutamic decarboxylase at pH 5 and glutamic deaminase at pH 8, but there seems to be no overlapping in the production of the two enzymes. In fact, at pH 6.5 to 7, little of either enzyme forms. This limited range of existence leads one to question the anabolic value of these processes and supports the hypothesis that the reactions are more valuable in permitting some degree of internal stabilization in an unfavorable environment. In this connection it will be observed that reaction (16) consumes hydrogen ions, thus raising the pH whenever a decarboxylase is formed and acts in an acid system. Correspondingly, deamination in alkaline solution tends to lower the pH according to

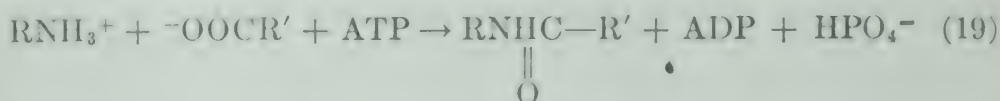


Hence, when the pH of the medium is high, deaminase is formed by the bacteria, and this enzyme locally lowers the pH by consuming hydroxyl ions in reaction (18).

### Proteins

The biological synthesis of proteins is an exceedingly important but little understood problem. It seems clear that ammonia is assimilated, amino acids are synthesized, and these are combined to form proteins. The general features of this combination are unknown. Organisms commonly possess

systems for the degradation of proteins, and it has been believed that the synthesis of proteins might occur by reversal of these degradations. Certainly such hydrolytic reactions can be reversed; however, free energy changes favor hydrolysis and the equilibrium would be in the direction of the smaller units. On the other hand, coupling to systems having a high energy may stabilize proteins and favor their formation. As a matter of fact, the formation of small peptides has been correlated with the phosphorylation of ATP.



Coenzyme A seems to be necessary, and it may be that an acylcoenzyme A complex is formed in the presence of ATP. This complex may then transfer the acyl radical to the amino group and produce a peptide. Enzymes certainly would be required for this process, and in studies with enzyme preparations from animal tissues it appears that these synthetic systems involve different enzymes than do the degradative systems.

Proteins must be broken down extracellularly by those organisms which use them as sources either of nitrogen or of amino acids because these molecules are too large to pass through the membranes of cells. Many species of bacteria produce proteases capable of hydrolyzing proteins, and these enzymes ordinarily are constitutive rather than adaptive, appearing whether substrates are present or not.

The study of the extracellular enzymes, especially the proteolytic types, is complicated by the elaboration of materials from dead organisms. The very enzymes which produce autolysis on the death of an organism may be the proteases which can be detected in aged bacterial cultures. On the other hand, a number of bacteria elaborate large quantities of proteolytic enzymes even in young rapidly growing cultures. For such species there is little doubt that these are true extracellular enzymes. They differ in their substrate specificity depending on the species, and all seem to be insensitive to oxygen. The extracellular proteases cleave various, but by no means all, proteins into peptides. At this stage the reaction seems to stop, but it has gone far enough to provide products whose size allows them to be assimilated.

In older cultures proteases appear which are sensitive to oxygen and they are accompanied by *peptidases*, enzymes hydrolyzing peptides to the individual amino acids. Presumably these two types of enzymes are of intracellular origin and appear in the medium only when cells lyse. When both proteases and peptidases are present, the substrate proteins can be completely hydrolyzed and provide amino acids for those organisms in the



culture that are still living. It is difficult to know whether such intracellular enzymes exist in active forms inside the intact living cells. If they do, then they may serve in the unending processes of protein synthesis and degradation. On the other hand they may be present in the form of inactive precursors which become active only when the cell dies and autolysis ensues.

### *Nucleic Acids*

The metabolism of nucleic acids and their component structures is now coming under active exploration. Much of this interest is due to a revival of work on the structure of nucleic acids and to a realization that at least in part the harmful biological effects of radiation involve changes in these compounds. It is still difficult to generalize from the published results because the structures of these large molecules have not been established and more important because one cannot readily distinguish between the nucleic acids from different organisms.

Bacteriologists have been concerned with nucleic acids inasmuch as these compounds make up a rather high percentage of bacterial substance. Furthermore, they have been implicated in both the genetic mechanisms and the synthesis of proteins by all cells. In view of the fundamental importance of these two processes the nature of the biological role played by the nucleic acids becomes a prime consideration. Therefore we will consider briefly both the chemistry and biochemistry of this group of substances.

Nucleic acids are structurally large and complex. Even the determination of their sizes has been a troublesome problem since they are subject to hydrolysis in *in vitro* systems. This lack of stability is especially pronounced in solutions that are either acidic or alkaline. Unfortunately, methods for separating nucleic acids from cells without the use of rather extreme pH values are not applicable to all situations. Hence the literature contains widely differing data on the molecular weights of nucleic acid preparations from a single species. Consequently, this situation makes it difficult to know whether different species possess nucleic acids of different sizes or even to determine whether any given species contains nucleic acids of many sizes. At any rate, the molecules are large, ranging from at least several thousand to perhaps two million in molecular weight.

As will be outlined these molecules contain a number of highly ionized phosphate groups. Thus they are isoelectric at low pH values, too low for precise measurement since the high acidity produces a rather rapid hydrolysis. Because of the strongly ionized phosphate groups, nucleic acids have a large number of negative charges when in solutions anywhere near or above neutrality. There is evidence that these large negative ions react readily with certain proteins, namely those containing an excess of strongly basic groups and which act as cations. The complexes or compounds formed



in this way are called nucleoproteins, and it seems probable that nucleic acids exist in such combinations inside ordinary cells. The amount of energy required to remove nucleic acid from a nucleoprotein varies with the nucleoprotein. Oftentimes the protein must be denatured before the nucleic acid can be split off. The term *protein nucleate* has been employed to designate those nucleoproteins from which the nucleic acid may be separated with relative ease, as, for example, by electrophoresis.

Methods are available for the isolation of nucleoproteins. However, these methods often involve the use of high concentrations of salts which would permit ionic exchanges and allow the nucleic acids to combine with proteins other than the particular kind to which they were originally attached in the living organism. After nucleoproteins have been isolated the nucleic acid

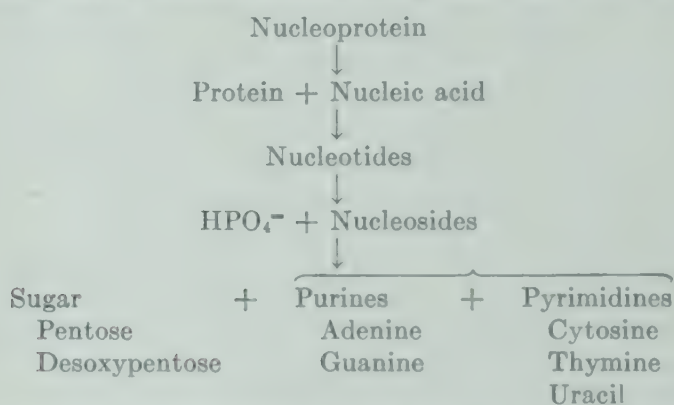


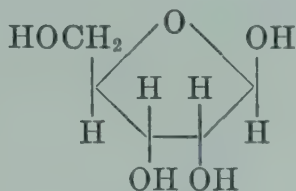
FIG. 90. A schematic representation of the degradation of a nucleoprotein through successive stages to the individual chemical components.

may be freed from the protein by any of the usual treatments leading to the denaturation of proteins. Of the various possibilities heat denaturation and surface denaturation by shaking with chloroform have been used most often.

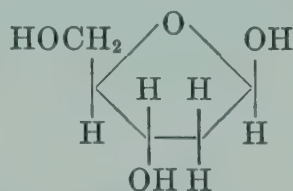
One of the serious experimental difficulties encountered in research in this field hinges on the lack of a method readily capable of distinguishing between nucleic acids of different structures. Unfortunately, these compounds exhibit no antigenicity nor do they act as haptenes, thus preventing the use of a powerful qualitative tool. Although it long has been known that two types exist (desoxypentose- and pentosenucleic acids), it is only recently that evidence reveals that the types may be further subdivided, perhaps almost indefinitely.

Most of the knowledge of the structure of nucleic acids is summarized in Figure 90 in terms of the names of the various components. The sugars obtained by the hydrolysis of nucleic acids are of two types, pentose and desoxypentose. Although there was no real evidence for the point of view, it was originally believed that only two different sugars, ribose and desoxy-

ribose, exist naturally in nucleic acids. While the hypothesis may be true no one has made a systematic attempt to verify it, and there is no definite knowledge of the nature of the carbohydrate in the nucleic acids except in a very few species of organisms. *Saccharomyces cerevisiae* contains a pentose-nucleic acid whose sugar is  $\beta$ -D-ribose, and the thymus of the calf is a common source of a desoxy-pentosenucleic acid containing 2-desoxyribose.

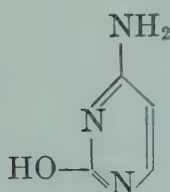
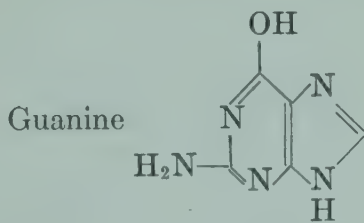
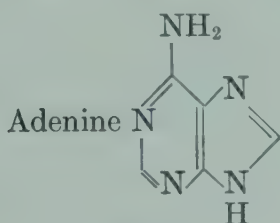


D-Ribose

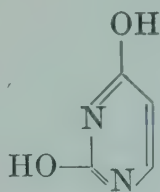
2-Desoxy- $\beta$ -ribose

These two carbohydrates may be quite widespread among nucleic acids, but the color reactions commonly employed in analyses by workers in the field cannot be regarded as sufficiently specific for purposes of their positive identification. As a matter of fact, the occurrence of xylose in one nucleic acid has been reported.

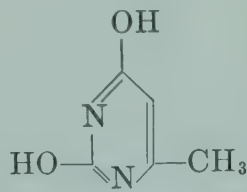
The heterocyclic structures which are now thought to be the common ones in nucleic acids are:



Cytosine



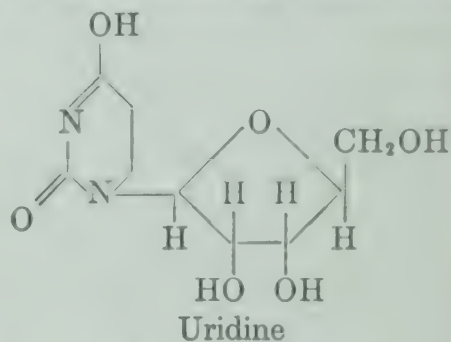
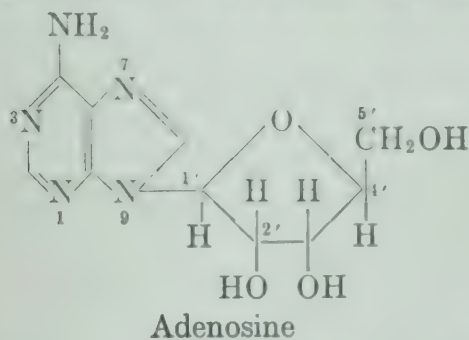
Uracil



Thymine

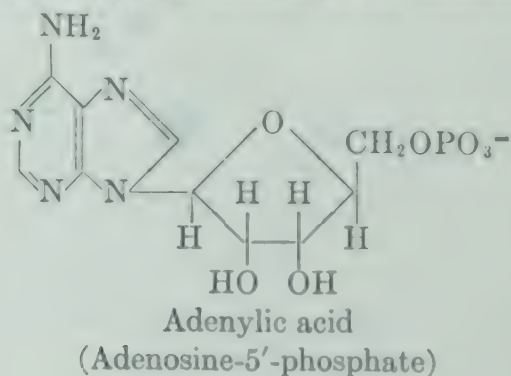
It will be apparent that all of these compounds are subject to tautomerism and will probably occur as equilibrium mixtures of the various possible structures. In addition to the above bases 5-methylcytosine has been reported in nucleic acid from the avian tubercle bacillus, but this observation has been challenged for this organism although the compound does appear to occur in animals. There have been several discoveries of heterocyclic materials of unknown structures in hydrolysates of nucleic acid preparations. Presumably then, not all of the purine and pyrimidine bases have been identified.

Nucleosides contain both a carbohydrate and a heterocyclic base. Quite a number of possibilities exist using only the well known sugars and bases since the manner of combining the two components might conceivably vary extensively. Actually the sugar seems always to be attached by way of the hydroxyl group on position one and is clearly a  $\beta$ -glycosidic linkage. The purines, adenine and guanine, are always linked through position nine, contrary to earlier opinions, and the pyrimidines at position three. Adenosine and uridine are taken as illustrations of the structures of nucleosides.



The other known nucleosides differ only in the heterocyclic portion or contain 2-desoxyribose although not all of the theoretically possible compounds obtained from these combinations are known. In addition to their occurrence in nucleic acids both of the structures shown above fill other roles. Adenosine makes up a part of ADP, ATP, DPN<sup>+</sup>, and TPN<sup>+</sup>. Uridine occurs in uridinediphosphoglucose, the coenzyme for the conversion of galactose-1-phosphate to glucose-1-phosphate.

The next larger compounds obtainable from nucleic acids are the nucleotides which may be hydrolyzed to phosphate and nucleosides. There is no evidence to indicate that the phosphate is ever attached to the purine (or pyrimidine) portion of the nucleotide molecule but is combined instead with the carbohydrate. Nucleotides containing adenine have recently been obtained from yeast ribonucleic acid corresponding to all three possible isomers, there being three hydroxyl groups on the ribose available for esterification with phosphate. A typical example is the nucleotide





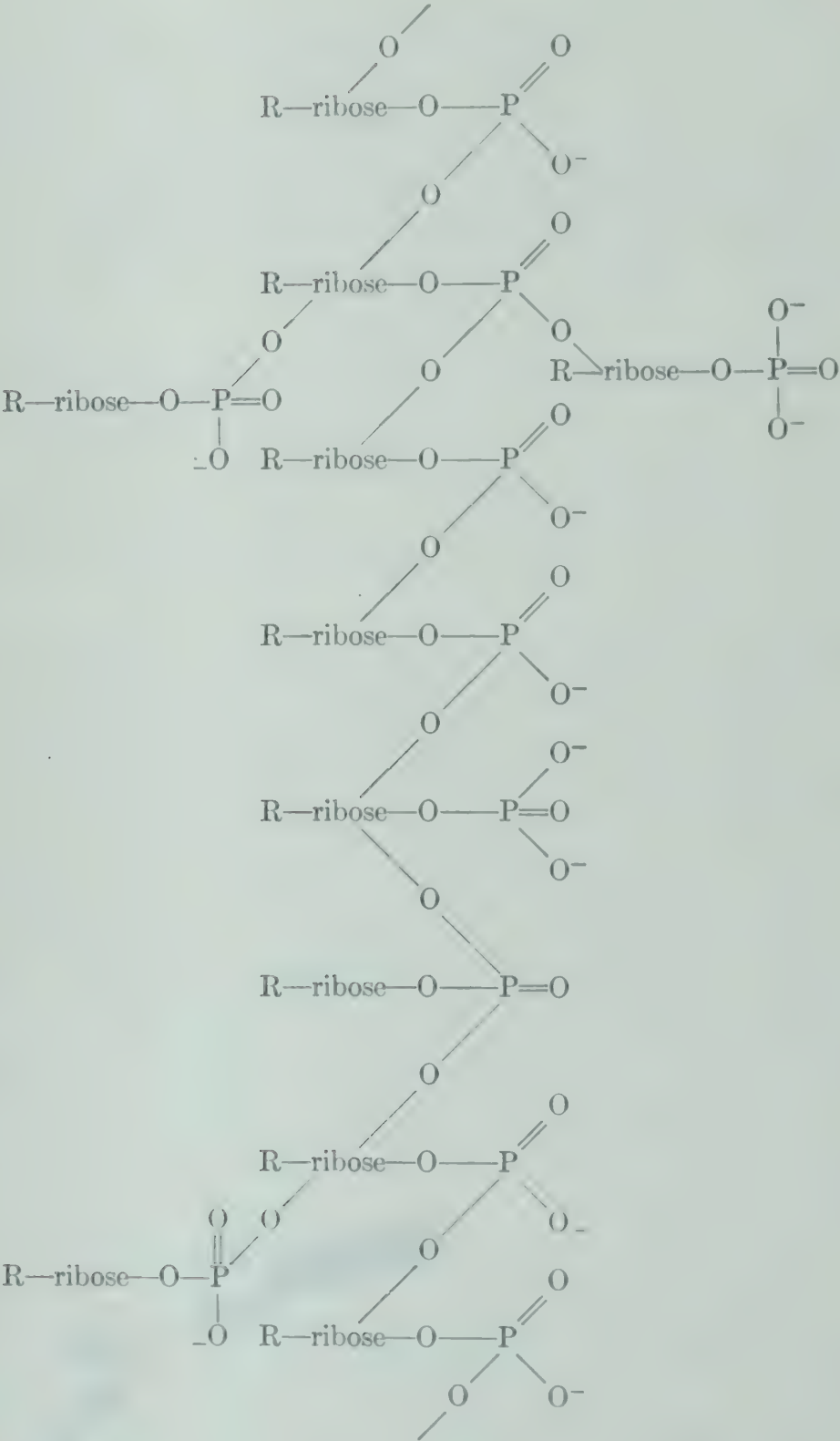
which has been isolated from enzymatic hydrolysates of calf liver ribonucleic acid. The other isomeric adenylic acids have been demonstrated in yeast and are the 2'- and 3'-phosphates. Many of the other nucleotides occur as more than one isomer, so the esterification of phosphate at more than one position seems to be rather common. Those nucleotides containing desoxypentose which have been examined seem to be 5'-phosphates only. However, these compounds have been isolated from enzymatic hydrolysates and it should be possible to prepare the 3'-isomers by using other methods of hydrolysis.

At this point it might be well to comment that the nucleic acids should probably be called nucleates since they exist as salts at ordinary biological values of pH. In any case the compounds consist of a number of nucleotides condensed to form large molecules. The great possibilities for structural variation are readily evident since three factors are operating. There are several different nucleotides which may be present and in varying amounts, the nucleotides may be united at various points, and the nucleotides of one kind may be all side by side or scattered in any manner within the nucleic acid molecule.

The structure of the ribonucleate of yeast has received the most attention and certain structural features have become apparent from titration curves and the isolation of the products of methylation of the nucleic acid. A section of the molecule has been represented by the following formula shown on page 562 where R indicates either a purine or pyrimidine. Several points of importance should be noted about this partial structure. First, each ribose is esterified in the 5' position with phosphate. Next, the large size of the molecule depends upon the presence of a chain composed of alternating ribose and phosphate units with none of the bases as a part of this chain. It will also be observed that some phosphates are present as monoesters, some as triesters, and most as diesters. The relative amounts in the three states have been established on the basis of titration experiments. In a similar way the ribose in addition to esterification at position 5' is frequently esterified at 3' and sometimes at 2' as well.

In the structure shown all the acidic hydrogens of the phosphates are written as ionized although it will be clear that the secondary hydrogens will not be ionized to an appreciable extent below a pH of 5. These secondary ionizations can occur only on those phosphates which are esterified with but one ribose unit.

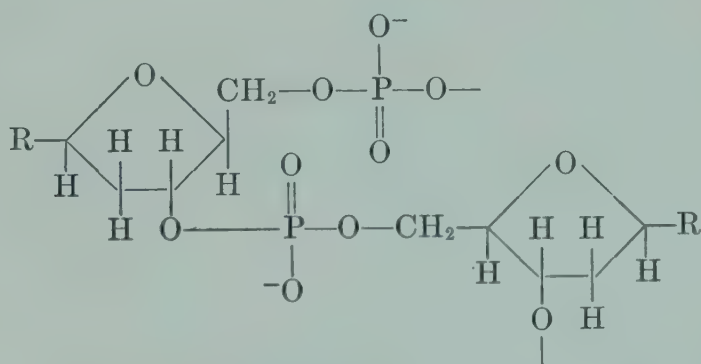
While the bases have been indicated without any attempt to identify them in the diagram presented, the location of the heterocyclic compounds is an important problem. The information available has been obtained mostly from studies of the hydrolytic action of ribonuclease. This enzyme degrades the nucleate into a mixture of mononucleotides, dialyzable polynu-



cleotides of low molecular weight, and a large limit nondialyzable polynucleotide not further attacked by ribonuclease. The mononucleotides contain only pyrimidine bases and actually represent a high proportion of the pyrimidines present in the original nucleate. The relatively small polynucleotides consist of the nucleotides of both purines and pyrimidines and are high in adenylic acid. Finally, the limit polynucleotide remaining unaffected by the enzyme contains the remaining bases and is high in guanylic acid, the nucleotide of guanine. This purine may make up two thirds of the total content of heterocyclic bases in this residual structure.

As a result of these and other observations it is felt that ribonuclease does not attack the bonds between guanylic acid and any other nucleotide. Hence the limit polynucleotide might contain adjacent or alternating guanylic acids. The pyrimidine nucleotides seem to occur largely in the side chains which are represented in the hypothetical structure as one membered branches but which are probably longer. In spite of these findings and the rapid progress being made in understanding the structure of yeast ribonucleic acid, the areas of ignorance are still of fundamental importance. Most important there is yet no basis for extending the concepts presented to pentosenucleic acids from sources other than yeast.

Little has been said of the structure of the desoxynucleates and little has been reported. It is felt that the constituent nucleotides are combined by means of ester bonds between phosphate and desoxyribose. In view of the structure of this sugar, branches on the chain would be unlikely since desoxyribose can only be doubly or singly esterified with phosphate as shown.



In opening a discussion of the metabolism of nucleic acids it is appropriate to comment on their role in protein synthesis. While it is clear that the pentosenucleic acids are involved the nature of the process is uncertain.

Cells which are actively growing and synthesizing protein contain an unusually high quantity of pentosenucleic acid, probably as nucleoprotein.



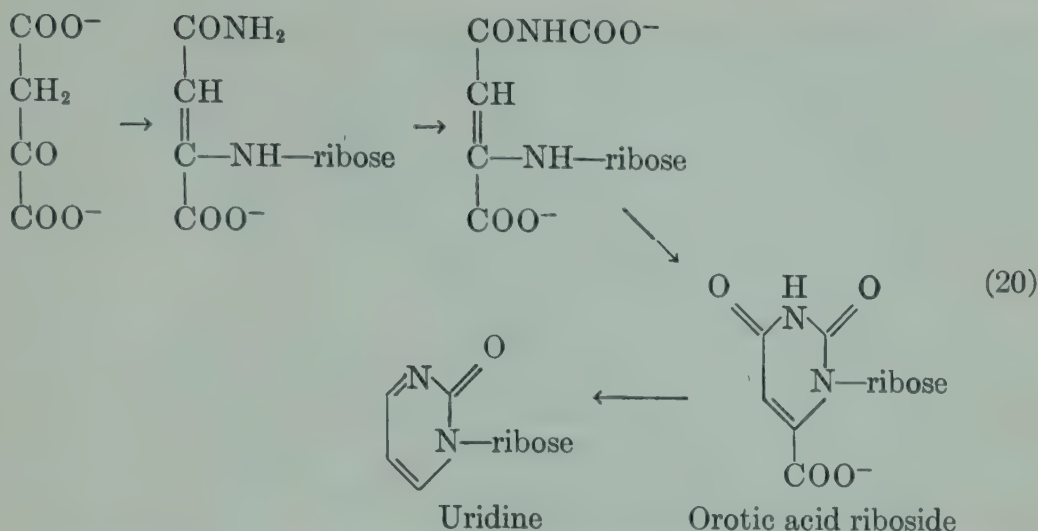
When the cells cease growing and are in a resting stage or metabolize without growth, the nucleic acid content is significantly lowered. Furthermore, the phosphorus of the nucleate is rapidly exchanged during the synthesis of protein as contrasted with retention of isotopically labelled phosphorus of the nucleic acid under other circumstances. It has even been shown that the formation of an adaptive enzyme involves the exchange of the phosphorus of the pentosenucleate with the medium and the participation of the nucleic acid itself although a net gain in protein does not occur.

These views have led to the hypothesis that the nucleate may participate in transferring the energy needed for protein synthesis and that the phosphorus plays an important part in the transfer. Certainly the nucleic acid could provide a reservoir of phosphate having some potentially available free energy. However, one would not expect this energy to be particularly high and hence not very effective as an energy donor for the formation of peptide bonds. It seems then that if high energy phosphate is essential some source other than nucleic acid must exist, perhaps metaphosphate as has been suggested. This conclusion does not gainsay the fact that the phosphorus of nucleate is reacting in some way, and an explanation of the role of the phosphate is needed.

Desoxypentosenucleic acid seems always to be associated with nuclear material and increases in total quantity when the nucleus divides. The desoxynucleate is undoubtedly present combined with protein. If it is responsible for genetic control, as is probable, the mechanism of the genetic activity is unknown.

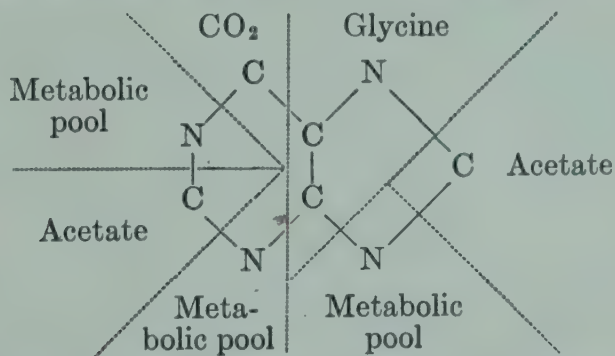
It is known that an increase in the pentosenucleic acid always precedes an increase in the desoxypentosenucleic acid. This relationship has led some investigators to believe that one may serve as a precursor for the other. However, it now appears more probable that the desoxypentosenucleic acid is built up from small units. In this connection multiplying *Escherichia coli* synthesizes three times as much pentose as desoxypentosenucleate. However, when the bacteria are infected with bacteriophage only the latter increases. Moreover, it has been shown that the phosphorus in the DNA (desoxypentosenucleic acid) comes from the medium and not from the PNA which, therefore, did not serve as a precursor for the DNA.

Although the origin of the heterocyclic bases is far from clear, a picture for pyrimidines is developing as a result of work with *Neurospora*, *Escherichia coli*, and *Lactobacillus arabinosus*. Oxaloacetate can serve as the carbon chain for the pyrimidines, and a number of nitrogen compounds seem to be involved as intermediates. Some of the observations may be combined very tentatively into the sequence of reactions shown.



The last step may not occur until the nucleoside is incorporated into the nucleic acid since uridine is not so effective for the growth of a deficient strain as is orotic acid which corresponds to the preceding structure except for the absence of ribose. Certain compounds having five membered rings may precede the nucleoside of orotic acid, requiring a modification of the above scheme. Once any one pyrimidine has been formed it may be transformed *in vivo* into the other derivatives. At any rate, a strain of *Escherichia coli* is capable of deaminating cytidine to uridine and hydrolyzes cytosine desoxyriboside even more rapidly. The enzyme responsible for the reaction appears to be rather specific, showing no action on the corresponding nucleotides. The existence of this type of reaction does suggest the possibility of the interconversion of closely related bases. There are almost certainly a large number of reactions participating in the synthesis of pyrimidines.

Knowledge of the origin of purines is in a somewhat similar state and may be summarized schematically. While this diagram



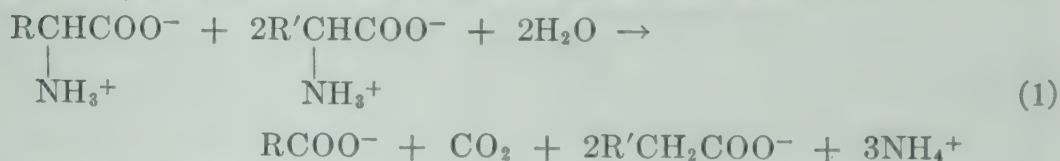
represents the known sources of the atoms of the purine ring system, one

cannot say that both adenine and guanine are derived solely in this way in all organisms. Actually, the present picture is a composite based on data taken with more than one organism. The carbon dioxide and ammonia may be provided by any reaction producing these substances. In rats at least, isotopic experiments reveal a rapid and reversible deamination of adenine, again indicating that the interconversion of bases may occur.

### *Stickland Reaction*

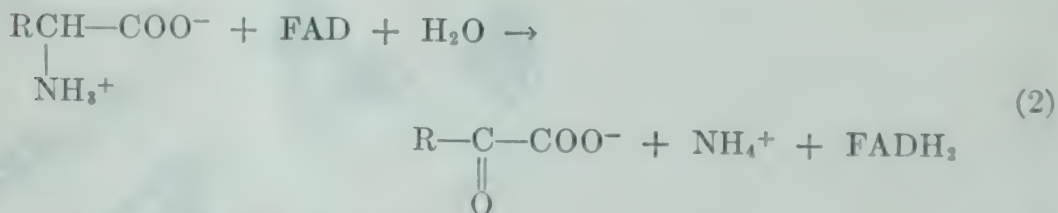
Certain species of anaerobic bacteria are able to grow in a medium in which amino acids are the only substances present in sufficient quantities to serve as energy sources. The organisms are largely, if not entirely, limited to the genus *Clostridium*. Of these species *Clostridium sporogenes* has been the subject of most of the investigations, and the present remarks will be based on data obtained with this organism.

In an amino acid medium free of fermentable carbohydrates certain of the amino acids serve the clostridia as hydrogen donors and others serve as hydrogen acceptors when oxygen is absent. It appears that the individual amino acids fall into either a group of donors or of acceptors but not both. In other words, two different amino acids are required, one from each category. The overall reaction, or as it is known, the *Stickland reaction*, seems to be the same in most cases and can be generalized as:



The hydrogen donor is oxidized first to an  $\alpha$ -ketoacid and then to the next shorter acid, and the acceptor is reduced with the total formation of three equivalents of ammonium. The two acids formed thus may be further metabolized by any mechanism available to the organism.

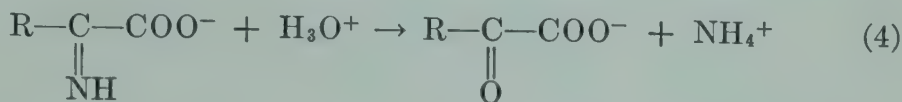
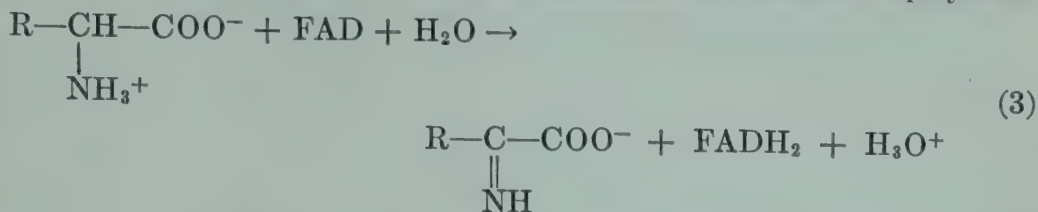
Present evidence suggests that the Stickland reaction (1) is composed of at least three steps of which the first involves a primary hydrogen (an electron) acceptor that is either FAD (oxidized flavinadeninedinucleotide) or something similar.



Reaction (2) is catalyzed by an enzyme called either amino acid dehydrogenase or L-amino acid oxidase. It will be observed that the actual net gain of two electrons and two protons by the FAD is all at the net expense of a



molecule of water. This situation raises questions of the suitability of the names of the enzymes. The difficulty is, however, probably more apparent than real since reaction (2) may be broken down into a two step system:

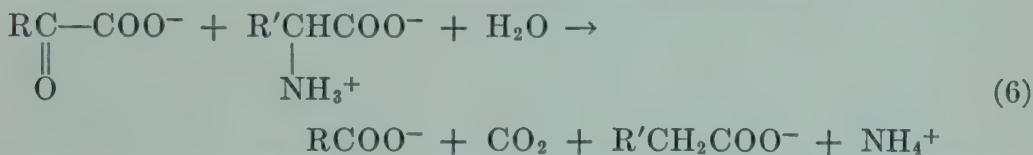


The enzyme actually would affect only reaction (3) which is followed perhaps spontaneously by the hydrolysis (4).

The second main step of the Stickland reaction consists of the reoxidation of the  $\text{FADH}_2$  (reduced flavinadeninedinucleotide) by the acceptor amino acid in the presence of amino acid reductase:

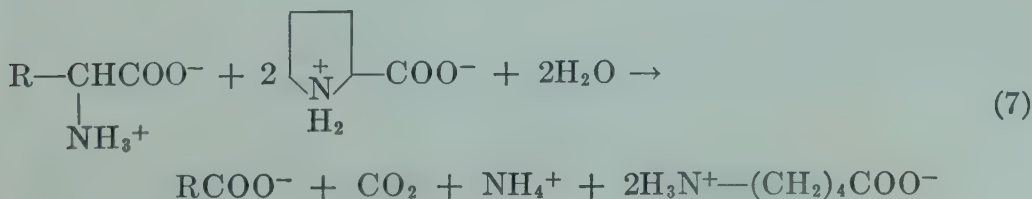


This reaction may run concurrently, precede, or follow the oxidation of the ketoacid of reaction (4) since the two processes are independent. Undoubtedly the reaction catalyzed by  $\alpha$ -ketoacid dehydrogenase



is composed of individual steps some of which have already been considered in connection with the oxidation of pyruvate to acetate.

*Clostridium sporogenes* is actually able to oxidize alanine to acetate, carbon dioxide, and ammonium while simultaneously reducing two molecules of glycine to two molecules each of acetate and ammonium. If proline is the acceptor in the oxidation of alanine, the reaction differs somewhat in that the ring structure is opened but ammonium is not formed from the proline.



The anaerobes using the Stickland reaction are also able to utilize amino acids of the hydrogen donor group in aerobic oxidations. Reaction (2) occurs and is followed by the usual steps of the Stickland reaction when an accepting amino acid is present. At the same time oxygen competes with this acceptor for the  $\text{FADH}_2$  and in part participates in the overall reaction system.



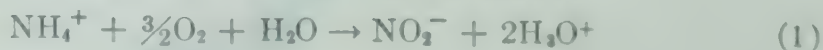
When an amino acid suitable for the oxidation of the  $\text{FADH}_2$  is not present then only reactions (2) and (8) of the Stickland system occur. The latter obviously cannot be participating under anaerobic conditions. On the other hand, the completely aerobic process does not result in ordinary growth of the clostridia.

### *Nitrification*

The bacterial metabolism of inorganic nitrogen may be divided into three more or less biologically separate processes. Of these *nitrification* results in the oxidation of ammonium ions (or ammonia) and amines to nitrate. Inasmuch as geochemists postulate that the atmosphere of the earth was reducing in character during the early stages of the formation of the earth, it has been proposed that early organisms used ammonia as a source of nitrogen. When free oxygen began to appear, autotrophic nitrifying bacteria evolved, capable of oxidizing ammonia to nitrate. Since such organisms diminished the pool of available reduced nitrogen and produced a new potentially available source of nitrogen, still other living forms developed to carry out the second process, *denitrification*. This step of itself was no real solution of the problem of maintaining the biological pool of nitrogen since the rather chemically inert product, molecular nitrogen, was formed. However, this stage must have been quickly followed by the evolution of species able to fix atmospheric nitrogen in the form of ammonium ions and other reduced structures, and thus return the nitrogen to the biological pool.

Although nitrification has fundamental agricultural and biochemical importance little is known about it. The detailed mechanism of the process has not been elaborated, so that only very general ideas may be presented.

When amino acids occur in nitrifying soils, they are split into ammonium ions and various organic acids, presumably by the typical deamination reactions of the microorganisms present in soil. The ammonium ions from this or any other source are then available for oxidation by the microflora. The bacterial species involved in the first overall stage of nitrification are known as *Nitrosomonas* and oxidize ammonium to nitrite:



The organisms seem to be most effective in carrying out (1) under neutral or slightly alkaline conditions, as might be expected from the appearance of hydrogen ions. Apparently ammonium ions are adsorbed on soil particles, and the bacteria multiply and remain near the sites of this adsorption.

In so complex a system as soil, the environmental factors can play important roles in controlling the overall results of a biological process such as nitrification. This truth probably explains the observation that prepared media never produce anywhere near the nitrifying activity that soils do. At least part of the difference must reside in the colloidal structure of soils since the addition of colloids considerably improves various laboratory media. Furthermore, most ordinary media contain a variety of organic substances that inhibit nitrification. In typical soils these inhibitors are utilized by heterotrophic species and adsorbed by the soil colloids thus giving the chemoautotrophic *Nitrosomonas* a greater freedom of action. One might anticipate from this reasoning that colloidal media with a mixed population of heterotrophs would facilitate the laboratory study of nitrifying organisms.

Once nitrite has become available the second and last stage of oxidation is brought about by *Nitrobacter*:

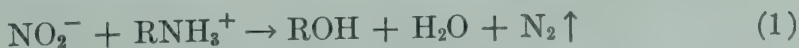


According to reactions (1) and (2) oxygen is consumed in both processes, and it is known that efficient aeration of soils facilitates nitrification. Actually it has been shown in manometric respiration studies that the theoretical quantities of oxygen demanded by this reaction are consumed during the oxidation of both ammonium and nitrite ions.

The nitrifying bacteria presumably obtain the essential carbon for their growth by fixation of carbon dioxide. For this purpose known mechanisms may suffice, but data on carbon dioxide fixation obtained with *Nitrosomonas* and *Nitrobacter* are not available.

### Denitrification

The denitrifying organisms reduce nitrate to nitrite or to molecular nitrogen and even to ammonia. When nitrite is the end product of the activity of one organism it may either be reduced further, thus completing the denitrification, or may be oxidized back to nitrate by some other species. In addition, there are purely chemical methods for removing nitrite, and under certain conditions these reactions assume potential importance in natural environments. Reaction (1) is cited as an example.



Any primary amine or amino acid will serve if the medium is slightly acid.



However, since most bacterial denitrifications occur in alkaline systems, this strictly chemical process probably does not often occur.

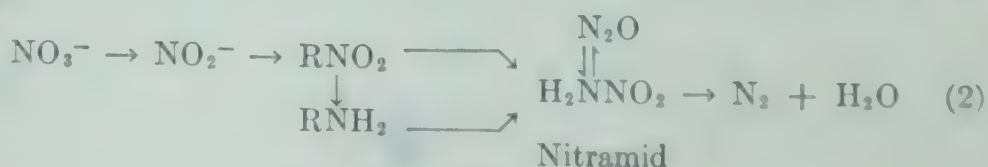
It was proposed in the early 1900's that the denitrifications were quite analogous to the ordinary aerobic oxidations of microorganisms. It was held correctly that nitrate merely replaced oxygen as the ultimate oxidizing agent and that practically all of the compounds serving as oxidizable substrates in the presence of air could support growth anaerobically if nitrate is present. Admittedly, both oxidants cannot be used by all species, but energy coupling mechanisms are definitely present in either reaction system. In other words, species of denitrifying bacteria are known which can grow as the result of the energy liberated when any of a variety of inorganic substances such as hydrogen, sulfide, sulfur, or thiosulfate are oxidized with nitrate as ultimate oxidizing agent.

Certain species of denitrifying bacteria can grow using either nitrate or oxygen as the oxidizing agent. If the cultures were obtained originally by anaerobic growth, both systems can function in such organisms and even do so simultaneously, although the rate of denitrification is somewhat suppressed. However, if the bacterial cultures were grown aerobically, no activity for nitrite or nitrate reduction exists when subsequently tested anaerobically. It would seem that the denitrification mechanism is formed adaptively and is blocked by a fairly high concentration of oxygen. At low oxygen pressures during growth of the bacteria some denitrifying activity develops, and the interference is only partial.

Many of the metabolic reactions of denitrifying species must be like those occurring during ordinary aerobic metabolism. Obviously, however, the actual reduction of nitrate must involve reactions peculiar to denitrifying systems, and we shall be concerned here with these processes.

The first known step in denitrification yields nitrite since with *Clostridium sporogenes*, for example, nitrite appears as a first product during the reduction of nitrate, then the nitrite disappears and ammonia accumulates. In addition, other organisms, for example some strains of *Escherichia coli*, are unable to carry the process beyond the stage of reduction to nitrite. This situation may then lead to reaction (1) when amino acids are present and the medium becomes acidic during growth.

Whereas this first reaction, the reduction of nitrate to nitrite, has been established rather conclusively, the subsequent stages of nitrate reduction have been in doubt. As a result of a recent reopening of the matter with the experimental technics now available a reasonable hypothesis is emerging:



In this system of reactions each step removing oxygen from a nitrogen compound apparently yields water, even the last at which hydrogen peroxide rather than water might have appeared but is not found at least with *Pseudomonas stutzeri*. Nitramid is an intermediate and may yield nitrous oxide reversibly. The latter is sometimes an end product but is not directly in the reaction chain between nitrite and nitrogen. The mechanism of the formation of nitramid is in doubt, but the suggestion has been made that the intermediate arises by the interaction of organic nitro and amino compounds. The amine could, of course, be formed by reduction of a portion of the nitro compound which in turn could be derived from nitrite by nitration of some cellular substance.

At present one cannot do much more than speculate concerning the reactions involved in those organisms which reduce part or all of the nitrate to ammonia. Those species capable of growing with the nitrate as the sole source of nitrogen also must reduce part of the nitrate below the level of reduction of molecular nitrogen. Suggestive evidence in this latter situation has been obtained with various mutants of *Neurospora* and indicates that reduction of nitrate does not proceed to nitrogen as in (2) or ammonia but yields an amino acid instead. From this starting point the other amino acids might be formed by means of the typical processes discussed earlier. Hence a part of reaction system (2) may be utilized and not proceed beyond the amino step.

This theory still leaves us without an explanation of the ammonia formed by *Clostridium welchii*. Is molecular nitrogen formed and then reduced by the mechanism of nitrogen fixation, is some intermediate in reaction (2) reduced to ammonia directly, or is the ammonia formed by deaminations of an oxidative or hydrolytic type?

### *Fixation of Nitrogen*

The process completing the biological cycle of nitrogen utilization involves the fixation of molecular nitrogen from the atmosphere. Obviously, the practical importance of nitrogen fixation to biology lies in the maintenance of the pool of available nitrogen, since without fixation, this element gradually accumulates in the atmosphere and cannot be recovered.

Although a considerable amount of effort has been expended in research on problems of nitrogen fixation, a great deal remains to be learned. The slowness of progress is due largely to experimental difficulties and to the use of limited approaches. Effective analytical tools are becoming available and acceleration in the work will become evident.

Use of the heavy isotope of nitrogen ( $N^{15}$ ) has led to recognition of fixing of nitrogen by a large number of species of both heterotrophs and autotrophs. The active members of the first group are found among the genera, *Azotobacter*, aerobic species, and *Clostridium*, anaerobic species.



Photoautotrophic fixation is accomplished by the root nodule bacteria classified as strains or species of *Rhizobium*, by the blue green algae, and by members of all the families of photosynthetic bacteria. The present fragmentary evidence suggests that all of these varied organisms fix molecular nitrogen by the same general method although there are differences in individual details.

Experimentally nitrogen has not been fixed in cell-free preparations obtained from biological material. Indeed, it has not even been possible to demonstrate the process in resting cells, and it has been found necessary to resort to studies with growing organisms. Only in this situation has fixation been observed. With the species of *Rhizobium* experimentation is even more limited, for these organisms are completely symbiotic, depending in some way upon the associated plants for their ability to fix nitrogen. Excised root nodules containing the bacteria in their seemingly normal environment are ineffective; hence the plant itself performs some essential function. The other nitrogen fixing species are not symbiotic so that the root nodule bacteria are unique in this regard. In spite of such an important potential restriction as symbiosis, it is felt that most of the nitrogen fixed biologically in nature comes from the symbiotic system.

A number of inhibitions influence the kinetics of nitrogen fixation although not much is known of the details of these inhibitions. When usual sources of nitrogen other than molecular nitrogen are present, these sources may be utilized instead of fixation. This competition is pronounced with ammonium ions and occurs with nitrite and nitrate after a period of adaptation. Some amino acids may act in a similar way but to a lesser extent. It appears that these alternate sources supply the organism with the needed nitrogen, and the fixation mechanism then just does not function.

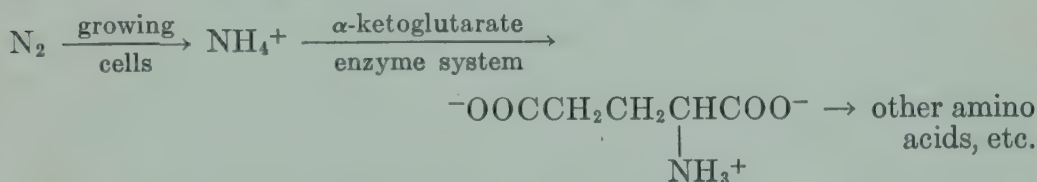
Some observations have led to the hypothesis that the reduction of nitrate and the fixation of molecular nitrogen have reactions in common. If true, then the common steps may be completely occupied by intermediates derived from nitrate so that fixation is blocked. A similar idea applies to the competition of ammonium except that this ion is thought to be one of the intermediates in ordinary fixation. Hence the presence of a relatively large amount of ammonium might saturate the enzyme systems and block fixation. Such a block could only occur if all steps between molecular nitrogen and ammonium are reversible. One logically would expect then that *Azotobacter* could form molecular nitrogen from ammonium ions when the system was free of nitrogen. Even in an atmosphere containing nitrogen there ought to be exchange between the gas phase and ammonium. Tracer experiments which could test this hypothesis have not been reported.

The literature contains much speculation on the possible mechanism of



nitrogen fixation. The various intermediates proposed include such things as ammonium, hydroxylamine ( $\text{NH}_2\text{OH}$ ), nitrogen hydrate ( $\text{N}_2\text{H}_4\text{O}_2$ ), oximes ( $\text{RR}'\text{C}=\text{NOH}$ ), and hyponitrous acid ( $\text{HONNOH}$ ). Good evidence exists supporting a role for ammonium in the system. Ammonia can be isolated, it can replace nitrogen, and in experiments with isotopes the distribution of heavy nitrogen in the nitrogenous compounds of the cells is the same for ammonium as for molecular nitrogen. The evidence indicating the existence of the other suggested intermediates is not as substantial although it is obvious that the reduction of nitrogen must be stepwise and through the participation of intermediates. The recent findings on the mechanism of denitrification will necessitate a revaluation of the hypothetical mechanisms of fixation if the two processes actually do have some portion of their metabolic pathways in common.

Once ammonium is formed tracer studies indicate rather conclusively that  $\alpha$ -ketoglutarate is converted to glutamate using the ammonium ion. Aspartate also may be formed directly from ammonium, but the other amino acids are formed for the most part by transaminations and the other metabolic reactions previously described. The established features of the general situation may be summarized by writing:



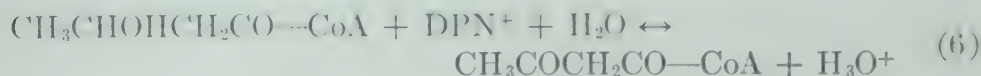
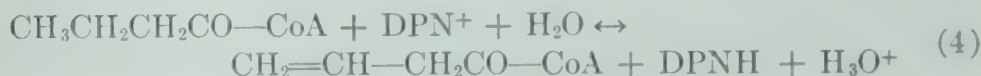
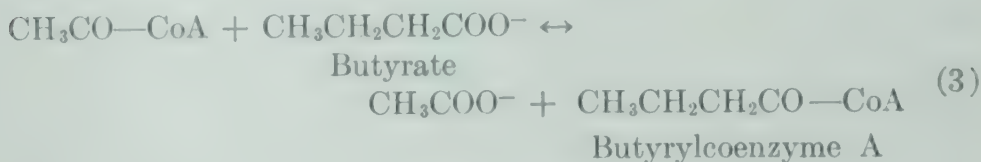
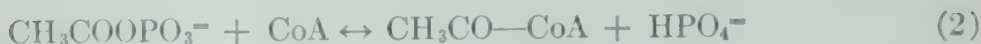
Among the various unsolved problems in the fixation of nitrogen is the function of a pigment resembling hemoglobin and which has been isolated from root nodules. This pigment is implicated in some unknown way in nitrogen fixation, but the substance occurs in the plant tissues rather than in the bacteria of root nodules and has never been found in *Azotobacter* and other free living nitrogen fixing species.

#### LIPID METABOLISM

Little is known of the lipid metabolism of bacteria, but a great deal of interest in the subject is displayed. Glycerol as a component of fats can be metabolized by many organisms by way of its connection with the metabolism of carbohydrates as discussed previously. Certain aspects of the mechanism of synthesis of fatty acids have also been considered under the products of fermentation. In addition the connection of fatty acids to the tricarboxylic acid cycle has already been indicated.

*Clostridium kluyveri* is able to catalyze both the oxidation of butyrate to acetate and acetyl phosphate and the reverse process as well. A speculative

mechanism based on various studies is shown in the following sequence of reactions.

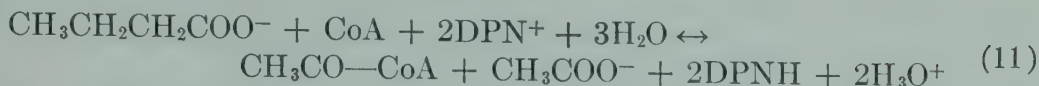


Reactions (2) and (8) are, of course, the reverse of each other revealing a net uptake of phosphate. The enzyme involved is phosphotransacetylase. The last step (9) merely connects to the better known high energy phosphate system. One might anticipate that acetylphosphate need not be involved more than momentarily at any time that ATP and acetate are present (see reaction (1)). Reaction (3) activates the butyrate by formation of the acyleoenzyme A compound which is oxidized in two stages, reaction (4) and (5). The first yields vinylacetylcoenzyme A which is hydrated to the hydroxybutyrylcoenzyme A compound, and this is dehydrogenated to acetoacetylcoenzyme A (reaction (6)). At this point dissociation may occur although this process



appears to be slow compared to the other reactions. Reaction (7) is the step during which the carbon-carbon bond is broken and would be expected to yield two molecules of acetylcoenzyme A. In addition to degrading the four carbon compound, this reaction results in the formation of a second high energy molecule.

The net overall process becomes



during which four electrons are transferred to  $\text{DPN}^+$ , and one high energy compound appears. The  $\text{DPNH}$  may be reoxidized by whatever electron transferring system the organism possesses. All of the various individual reactions probably are catalyzed by enzymes although the active enzymatic extracts of organisms so far studied have not been separated into the various individual components.

Since butyrate may be synthesized as well as degraded, all of the above reactions have been written as reversible. The individual reactions of the mechanism postulated either must be reversible or different pathways must exist for synthesis and for degradation.

The general reactions of which (3) to (7) are special cases could account for the metabolism of the longer chain fatty acids. These compounds are oxidized apparently at the beta carbon positions, and acetate is eliminated. A fatty acid two carbons shorter than the parent compound results, and the process is repeated until the degradation has been completed. There is evidence suggesting that the mechanism is probably more complex for the longer chain acids, but the situation awaits clarification.

Glycerol is oxidized by many heterotrophic bacteria and may also serve as the sole source of added carbon for many of the species that do not require particular organic sources of nitrogen. The addition of glycerol to media will often result in the prominent appearance of lipid inclusions in bacteria. Cytological examination of aerobic spore-forming bacteria grown on media containing glycerol in place of carbohydrate leaves the definite impression of a greater accumulation of lipid inclusion bodies in the bacteria grown in the media containing glycerol. If any future collection of quantitative data on the relative neutral fat content of these bacteria grown under various conditions were to substantiate such an observation, the direct utilization of extracellular glycerol in the synthesis of neutral fats would seem to be a distinct possibility. The promoting effect of glycerol on inclusion formation is not limited to fat formation since the production of metachromatic granules by *Corynebacterium diphtheriae* is also favored.

As a nutrient, glycerol may have an advantage over carbohydrates in the greater ease with which it penetrates into cells. While no data can be quoted for the eubacteria, studies of the permeability of *Beggiatoa mirabilis*, a species of *Chlamydothales*, show the following threshold values of plasmolysis in moles per liter of various polyhydric alcohols and carbohydrates: glycerol, 0.009; erythritol, 0.001; mannitol, 0.00055; arabinose,



0.0008; glucose, 0.00055; sucrose, 0.0002. It is well known that the human type of tubercle bacillus grows more luxuriantly on media containing glycerol than does the bovine type. It would be interesting to know whether this difference in growth is due to differences in the permeability these two organisms show toward glycerol.

#### METABOLISM OF MOLECULAR HYDROGEN

It is well recognized that the intermediate metabolism of hydrogen is of great importance, and much is known concerning it. Some aspects of this knowledge have already been covered in the discussions of energy transfer and in connection with photosynthesis. The remainder has been summarized as part of the general problem of intermediate metabolism. Thus no effort will be made here to deal separately with the entire problem of hydrogen, but rather the treatment will be limited to molecular hydrogen.

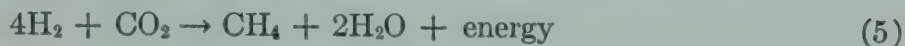
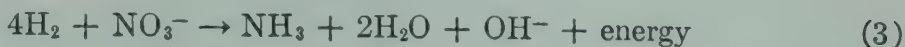
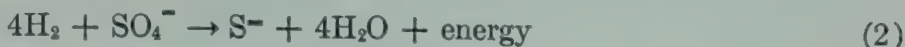
This problem has two aspects. Hydrogen is both a reactant and a product; it is sometimes consumed and sometimes produced. Dealing first with the utilization of hydrogen, it is recognized at the outset that a source is necessary. This need is not met as simply as might be first thought, for in biological situations with their mechanisms for reducing oxygen, hydrogen does not normally occur in appreciable quantities in the presence of molecular oxygen. Ubiquitous as this latter element is we must, therefore, seek hydrogen in localized zones of anaerobiosis or in the still less likely situations in which hydrogen is set free into the air.

The condition of anaerobiosis itself does not provide hydrogen, so accompanying it some process for the production of hydrogen must exist. Generally, this production is accomplished by the activities of living organisms rather than by inorganic processes which are ordinarily too slow and require too stringent conditions to permit the existence of life. Thus the utilization of hydrogen by a bacterial species is closely dependent upon other organisms which release it.

Molecular hydrogen may serve two purposes as a reactant. It may be incorporated into the cellular components by synthesis, or its oxidation may provide energy for the various processes of the organism. The latter is probably important in all organisms utilizing hydrogen, and the former is also likely to occur at the same time. Inasmuch as the synthetic uses of hydrogen presumably involve the typical metabolic reactions already outlined, the reader is referred to the section on intermediate metabolism.

In order to derive energy from hydrogen, oxidizing agents are required. Of the potentially available materials, oxygen, sulfate, nitrate, and carbon dioxide may be listed as established oxidants in bacterial systems. The

overall reactions may be written as:



These reactions are all carried out by bacteria, but as far as is known no single species can use more than one. Some are strict aerobes, e.g., species of *Hydrogenomonas* using reaction (1); others like *Sporovibrio desulfuricans* (2) are obligate anaerobes. *Escherichia coli*, though a true heterotroph, is still able to oxidize molecular hydrogen, and indeed this ability is found even among obligate aerobic heterotrophs. An enzyme named hydrogenase has been described in all these various organisms and serves to activate hydrogen, thus permitting its oxidation.

For most of the species capable of oxidizing molecular hydrogen this ability is probably not used except under rare circumstances. It might seem, therefore, to represent a rather useless system. However, evidence is being accumulated that suggests a role for hydrogenase in normal metabolism in the absence of hydrogen. It is conceivable, for example, that this enzyme links together the metabolism of both carbohydrates and amino acids or in other cases may function in photosynthesis or nitrogen fixation. Hydrogenase may serve the organism wherever hydrogen is formed by other reactions, or it may assist the survival and growth of the organisms fortunate enough to possess it when they are trapped in anaerobic muds. For a few species the oxidation of hydrogen apparently is an essential reaction (ordinarily by means of oxygen) and these are collectively called *Knallgasbakterien*.

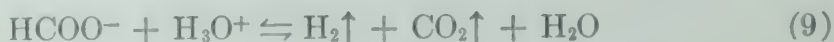
Turning our attention now to the production of hydrogen, it is well to consider hydrogenase once more since this enzyme can affect a reversible reaction. As a matter of fact, hydrogenase plays an important role in the liberation of hydrogen by the *Enterobacteriaceae*. A number of these species under certain cultural conditions break down carbohydrate partially into hydrogen and carbon dioxide through formic acid as an intermediate. Although not all conditions suitable to bacterial growth permit this reaction, it appears to be of some importance. Knowledge of the mechanism may be summarized by:



Reaction (6) is catalyzed by formic dehydrogenase and is connected by an intermediate electron carrier system probably containing iron to the step leading to the evolution of molecular hydrogen



in which hydrogenase is active. The overall effect, obtained by summing these three reactions, is



There is some evidence for the existence of a parallel but different mechanism for hydrogen production in various coli-aerogenes organisms; however, the process is not understood and seems to yield rather less of the gas than does the scheme outlined above.

Among species of *Clostridium* other pathways are clearly indicated since formate can be excluded as an intermediate.



is a typical overall reaction for the breakdown of pyruvate. In other cases amino acids are utilized and hydrogen is formed, usually with the concomitant appearance of carbon dioxide, ammonia, and organic acids. When cultural conditions are properly altered, the routes followed by metabolism shift, so that the evolution of hydrogen is much diminished. The presence of cyanide, carbon monoxide, and a deficiency in iron are among factors decreasing the formation of the gas, and all indicate that the metabolic evolution of hydrogen gas depends in some way upon systems that contain iron.

#### MECHANISM OF PHOTOSYNTHESIS

Photosynthesis has been discussed in general terms in the chapter on nutrition, but the consideration of mechanism has been delayed in order that advantage may be taken of the information on metabolic reactions presented in the preceding sections. The phenomenon of photosynthesis may be divided for convenience into two stages. The first involves the processes occurring when light strikes the photosynthetic organism and is absorbed and converted into chemical energy. This system might be called the light reaction in the sense that its activity depends directly upon light absorption.

Clearly this stage is of primary importance in photosynthesis and though much attention has been devoted to it, much remains to be learned. Infor-



mation available on certain points may be summarized as follows. A pigment such as chlorophyll absorbs radiant energy with a resulting increase in its own energy. Somehow the activated chlorophyll cleaves water molecules forming free and activated hydrogen and hydroxyl radicals. Perhaps the splitting of water takes place some time after the activation of the chlorophyll or it may be that there are several intervening steps, all essentially dark reactions in the activation of water. It is also possible that all steps are so rapid as to be nearly instantaneous.

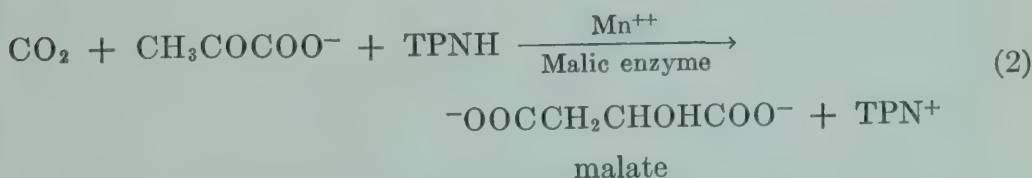
The processes succeeding the splitting of water can certainly take place without the need for radiant energy and are better understood than the purely photochemical events. As has been mentioned the hydroxyl radicals combine and yield water and oxygen in those organisms possessing catalase and similar enzymes. In the photosynthetic bacteria hydroxyl is reduced by means of electron donors such as sulfide, sulfite, hydrogen, depending on the species, so that oxygen is not formed as one of the products of photosynthesis.

In either case the activated hydrogen, however it may exist chemically, initiates the sequence of reactions leading to the reductive synthesis of cellular components from carbon dioxide, ammonia, phosphate, and the like. The first known appearance of the hydrogen from water in a relatively stable compound is in TPNH or DPNH according to



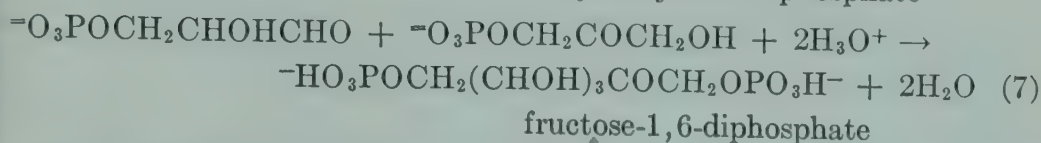
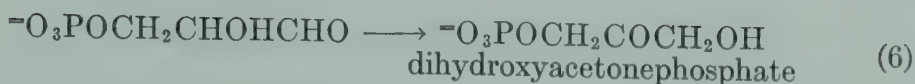
DPN<sup>+</sup> is reduced in the same system. The green grana are particulates containing chlorophyll and are readily prepared from the chloroplasts of spinach leaves. While this particular reaction may not be universal in occurrence, this general type of reduction process is assumed to be widespread even though active particulates have not been reported in photosynthetic bacteria.

When pyruvate and carbon dioxide are available the TPNH is reoxidized, and the carbon dioxide is fixed in the presence of manganous ions and malic enzyme:

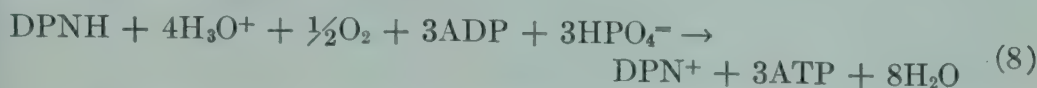


This reductive carboxylation appears to be a typical means for fixing carbon dioxide in both autotrophic and heterotrophic species. Another similar





Reaction (4) introduces a new requirement, that for ATP. This substance may become available through



which is essentially an assumed reversal of the overall process of oxidation of DPNH coupled to phosphorylation as discussed in an earlier section. While it does appear that phosphorylation can be coupled with the oxidation of DPNH, that fact does not mean that it actually happens in photosynthesis. This same qualification applies to each step of the proposed pathway for photosynthesis. Some progress has been made in this connection indicating that the normal reactions of respiration may indeed be involved at a few steps. Hence, studies testing the applicability of other portions of the respiration system are under way.

If photosynthesis and respiration do happen to utilize the same or many of the same enzyme systems, do the two processes occur simultaneously when the organism is irradiated? Differences of opinion exist on this point, some investigators believing that both processes take place and others that photosynthesis inhibits respiration. Resolution of the disagreement may depend upon establishment of the metabolic and photosynthetic pathways of different organisms.

The efficiency of photosynthesis has been investigated extensively from many points of view. On the one hand efficiency is low when based on crop yields of cereal plants over an entire growing season under field conditions, although the cultivation of algae in tanks under more effective conditions of nutrition may fix a higher proportion of the radiant energy. On the other hand, laboratory studies with the most favorable conditions devised have demonstrated that a high efficiency of upward of 65 per cent can be achieved. In fact, the use of intermittent irradiation has revealed that even more carbon dioxide can be fixed during the dark than during the light periods. This phenomenon is interpreted to mean that certain dark reaction systems set in motion by events occurring during the intermittent periods of illumination are able to supply sufficient energy to fix a large proportion of the total fixed carbon dioxide. Thus it well may be that photosynthesis is a very effective process when carried out under idealized conditions for the conversion of radiant energy to chemical energy.



## OPTICAL ISOMERISM IN BIOLOGY

It is a well known general phenomenon that living systems possess a high degree of specificity toward the optical configurations of compounds. The proteins are made up of amino acids all belonging to the L-family. The number and nature of the carbohydrates in a species is characteristic, and metabolically one enantiomorph (optical isomer) is employed to the exclusion of or at least to a much greater extent than the other. For example, the germination of endospores of various species of *Bacillus* requires a supply of amino acids. The specific amino acid requirement varies with the species, but those required belong to the L-family. In fact the presence of D-alanine strongly inhibits spore germination by its interference with the metabolic role of L-alanine, one of the essential amino acids.

Other cases are known in which the block imposed by the presence of the unnatural isomer of a compound can be avoided by some metabolic device. If L-asparagine is taken as the nitrogen source for certain brucella, growth occurs at once, smooth colonies are formed, and glutamate, valine, and perhaps other amino acids accumulate in the medium. When D-asparagine is included in the medium the growth of smooth colonies is suppressed and rough variants become preponderant. In addition, alanine appears in the medium although this amino acid is not observed with L-asparagine. Not only does the population change in its metabolic character but also in genetic character. Apparently the genetic change rests upon the selection of mutants by suppression of the growth of the parent smooth forms.

Specificity in the action of drugs is indicated in work with optical isomers, one generally proving more effective than the other. It appears that this and the other evidences of specificity have their origins in the asymmetry of the functioning enzyme systems of organisms. Given the original asymmetry of an enzyme one might expect a higher activity on one optical form of the substrate than on the other, owing to a need to fit the substrate to a particular spatial configuration on the enzyme.

However, the very existence of such specificity raises some thorny questions. Does this type of specificity offer advantages and of what kind are they? How is the separation of optical isomers accomplished and how is it maintained? Regarding the first point, advantages for optical specificity and optical purity have been postulated and as will be seen are quite reasonable although they have not been proven.

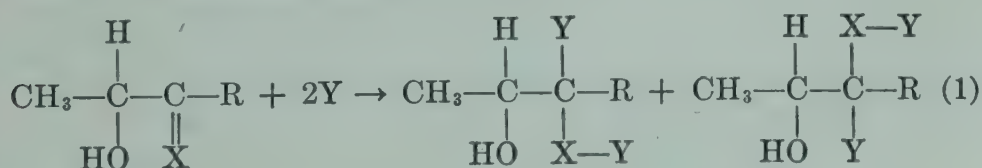
In considering these problems it is well to recall that optical isomerism is a chemical rather than a biological property and would exist in the absence of life. Therefore, an organism is presented with two optically isomeric forms of a compound as a result of purely chemical processes. Actually one isomer may be somewhat more abundant than the other as

will be shown later, but the essential problem of the possibility of use of either one or both isomers remains.

If one assumes that an organism possesses two systems of enzymes each able to metabolize only one of the particular forms of the substrate, the necessity for an additional structural complexity of such an organism may be readily conceived. Size may certainly be affected, the requirements for trace metals, coenzymes and the like would be increased, and perhaps most important of all the closely coupled reactions in the metabolically active particulates of the organism would be complicated greatly by the presence of parallel enzyme systems. Moreover, on a kinetic basis reactions would be slow because half of the collisions between specific enzyme and substrate would produce inactive substrate-enzyme complexes of L-D or D-L types. Full catalytic action can occur in our hypothetical organism only with D-D or L-L complexes.

Evidently the autotrophic species could derive no advantage whatever from the double enzyme system since the nutrient compounds withdrawn from the environment are simple, optically symmetrical substances. Therefore, the synthesis of cellular materials would be most rapid and efficient if optical specificity were maintained at all stages where molecular asymmetry appears. In the case of heterotrophs utilizing isomeric substrates one specific enzyme sequence would be kinetically as effective on a DL-mixture as a double arrangement and, moreover, would conserve cellular space and required cofactors. True, an optically specific heterotroph could not use the second isomer at all. However, present day heterotrophs get their substrates as a result of the metabolic activities of autotrophs and other heterotrophs, and these nutrients are optically specific materials. In such a situation a heterotroph with two sets of enzymes would not have substrates available for both.

The majority of chemical reactions leading to asymmetry yield essentially equal quantities of the isomers. Some reactions do not, however. If a molecule already possesses an unsymmetrical atom, the introduction of asymmetry at an adjacent position frequently results in an unequal distribution of the two isomers. Furthermore, these isomers are not mirror images of each other and have different physical properties. This idea may be clarified by means of a generalized example



in which  $\text{CH}_3\text{CHOH}$  represents any group containing an asymmetric atom adjacent to the unsaturated carbon atom. In reaction (1) one isomer can be



formed more rapidly than the other because the adjacent asymmetric group affects the double bond in some way so as to partially guide the reaction.

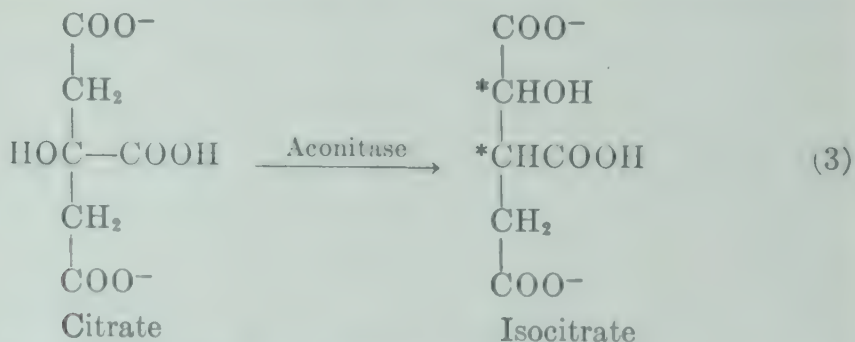
The configuration of the original group is maintained in (1) and makes the formation of mirror images impossible, for if we assume that the original molecule is dextrorotary then we obtain



and the products, therefore, do not have opposite configurations. In such cases the properties of the products of reaction vary; for example, one of the isomers may crystallize out of solution.

This general behavior is involved in many types of situations. The resolution of racemic mixtures by the formation of salts with optically active reagents is a related case. Catalyses in which symmetrical reactants yield optically active products in the presence of specific asymmetric catalysts are common examples. Here the reactant forms a complex with a specifically oriented catalyst which directs the reaction toward one of two configurations. After the reaction is complete the products dissociate from the catalyst but retain their optical specificity. The implications of this possibility to biology are enormous in view of the dependence of biological systems on enzymatic reactions.

A classical illustration of the formation of one optical isomer from a symmetrical reactant occurs in the tricarboxylic acid cycle. Citric acid is a symmetrical molecule but is converted by aconitase to the unsymmetrical isocitric acid.

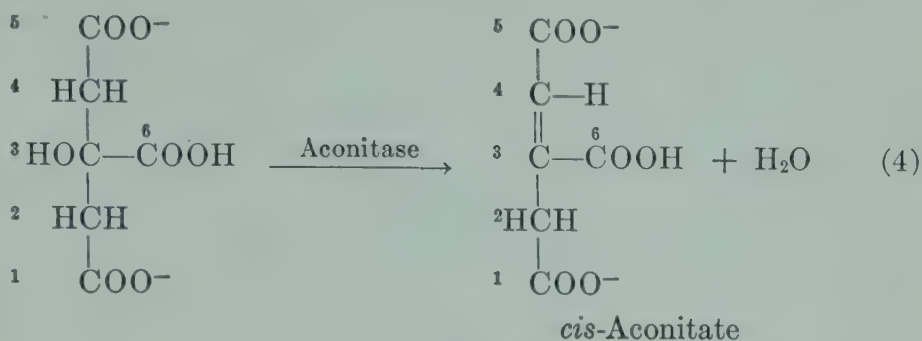


The asterisks in equation (3) denote asymmetric carbon atoms, and since two such atoms appear in the product four possible isomers theoretically could be formed. However, only one isocitrate is produced by organisms indicating that the directive influence of aconitase is optically specific. Obviously, the enzyme controls the orientation of the water added to the double bond of aconitate.

A second sort of asymmetric behavior also involving citrate was so puzzling as to disconcert students of metabolism. This problem arose in the dehydration of citrate to aconitate during which the two ends of the

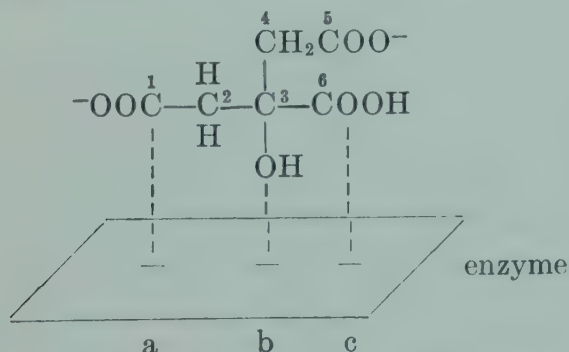


citrate ion actually appear to be different. The net reaction may be expressed by

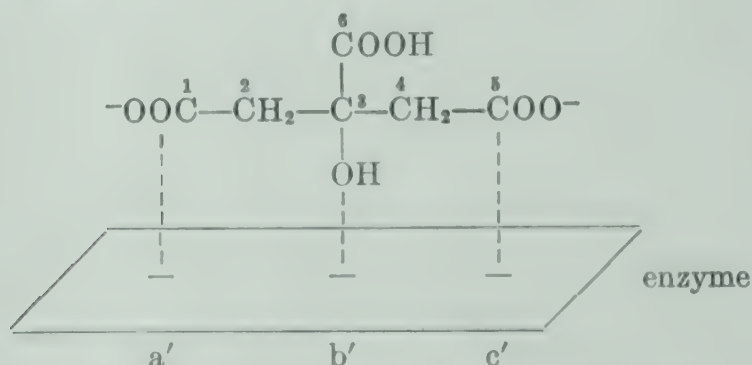


One would ordinarily expect that the elimination of water would remove the hydroxyl from carbon three and hydrogen from atoms two and four in equal amounts since the structure as written is symmetrical about a plane through carbon atoms three and six. Actually as shown by tracer experiments in which labelled citrate is formed in an organism by condensation of oxaloacetate and labelled acetate, citrate behaves as though carbons one and five differ. In these experiments citrate labelled at one end is formed as a complex in association with the condensing enzyme. In this complex the citrate is not symmetrical if it is attached to the condensing enzyme at three or more points whose catalytic properties differ. This unsymmetrical complex may then transfer citrate in a specifically oriented manner to aconitase with the result that the citrate is bound to the aconitase in an unsymmetrical way. Due to dissymmetry in the citrate-aconitase complex dehydration is completely oriented with respect to carbons two and four. The existence of this orientation is demonstrated by the finding that the isotopic atom bears a single fixed geometric relationship to the hydrogen atom lost.

The foregoing remarks may be amplified diagrammatically. Assume that three points of contact between enzyme and substrate are necessary and that catalytic differences occur at these sites:



Citrate is attached as in the above illustration to sites a, b, and c of the enzyme, and thus only dehydration between positions two and three would occur. This sort of complex could arise if only one enzymatic site is capable of attaching to a carboxyl attached in turn to a methylene group in the citrate ions. The other type of possibility is:



In this situation carbons one and five are both attached to the enzyme, but catalytic activity is exerted only between  $a'$  and  $b'$  since  $a'$  and  $c'$  differ in their catalytic effect.

Thus may dissymmetry be manifested by a symmetrical compound as was first suggested by Ogston. Ordinarily this particular type of phenomenon is not observed even when it does occur, unless two conditions are met. First, an indicator like an isotope must be introduced, and second, the introduction must occur in an unsymmetrical system, such as an enzyme-substrate complex which maintains a specific orientation.

Let us return for a moment to a consideration of non-living systems and discuss a spontaneous reaction that involves the formation of a center of optical asymmetry. In the absence of any directing influence both isomers will be produced in equal quantities and at equal rates because the process is purely statistical. However, when an optically directing catalyst is present one isomer appears at a greater initial rate than the other, but the second isomer is still formed as a result of the spontaneous reaction. The latter is true since most enzymes can be shown to increase the rate of reactions otherwise capable of occurring without the intervention of the biological catalyst. If an exceedingly effective catalyst promotes the formation of a metabolite of one optical configuration without affecting the rate of formation of the other isomer, then the first isomer is continuously made available in larger amounts for utilization in succeeding metabolic events. Although this arrangement could result in a high metabolic efficiency, it is not perfect because the spontaneous reaction yields a product which cannot serve as a reactant in optically specific enzyme systems. The accumulation of the unnatural isomer would be serious if the isomer were

toxic, and it would actually interfere with living processes. Consequently, the maintenance of optical purity in biological systems must also deal with the problem of disposal of unwanted isomers.

Two corrective mechanisms may operate. The simplest involves the provision of systems that specifically destroy the biologically undesirable enantiomorph. For the amino acids this may be the function of the dissimilatory D-amino acid oxidases which are widespread in their occurrence. The products subsequently could be reaminated into L-amino acids by enzymes having the proper specificity. While such mechanisms could function with the smaller molecules, there is no evidence that such a solution is possible for preserving the optical purity of large structures containing many centers of asymmetry. For these cases it is proposed that specificity is cumulative as will become evident.

In catalytic systems the excessive formation of one isomer is greatest at the start of the reaction and decreases slowly until equilibrium is reached at which point the product is racemic. These changes occur because both the catalytic and spontaneous processes are reversible and have the same equilibrium constants. With enzymes the catalyzed reaction is several orders of magnitude faster than the spontaneous reaction and if interrupted long before equilibrium is achieved a rather high optical purity can be obtained. The typical open reaction systems of biology prevent equilibration and effectively provide the equivalent of continuous interruptions favoring optical purity.

This situation is reinforced when a second specific enzyme introduces a second center of asymmetry into a process as in the condensation of two optically active amino acids to form a peptide. In such a case the amino acids are present in assumed ratios of 1000 parts L-form to one part D-form as the result of the original optically oriented synthesis. The coupling system for peptide bond formation, if of the same optical specificity as the synthesis of the amino acids, would yield the L,L-dipeptide almost completely ( $10^6$  to 1). Each succeeding step thus would swiftly increase the optical purity toward a state of perfection. Plainly the unnatural forms would tend to accumulate at the first stage of protein synthesis but the decomposition mechanism already mentioned could dispose of them at that level.

The possibility of optical purity depends for its existence upon the preponderance of one isomer over the other since the common methods of asymmetric synthesis depend upon the directive effect of an existing center of asymmetry. Biologically one organism depends upon its ancestors or on some other species for its optically active compounds. The given organism is then able to use these agents catalytically to make still other optically active substances.

Philosophically, however, we are faced with the theoretical problem of



the origin of the excess of the first optically active isomer. One possibility that may be tested experimentally depends upon the possible interaction of circularly polarized light with compounds undergoing reactions to produce asymmetric configurations. The unequal influence of such radiation does yield a slight but definite excess of one enantiomorph over the other. In the laboratory this result has been repeatedly obtained, and it is recognized that the method is capable of specific control. In other words, in a given reaction influenced by light circularly polarized in a given direction the isomer in excess is always the same. A portion of the radiation at the surface of the earth is circularly polarized, and the origin of asymmetric syntheses has been attributed to this fact. However, the fraction of such unsymmetrical radiation in the total radiation is quite small; hence, the quantitative effect on a reaction under natural conditions would be expected to be slight.

Therefore another solution has been sought, one based on a statistical effect. Although ordinary chemical reactions produce no measurable preponderance of one enantiomorph, probability theory indicates that rarely would two isomers be formed in exactly equal numbers of molecules. In fact it has been computed that there is an even chance of obtaining at least an excess of 0.021 per cent of one form over the other in every reaction producing an asymmetric atom. This might actually lead to distributions as uneven as those produced under natural conditions of radiation. On the other hand, a given reaction will yield a statistical excess of one isomer on one occasion and of the other isomer on the next occasion. Optical specificity might thus arise but would not be universal, the opportunity would be afforded for evolution of some organisms able to use one enantiomorph and of other species able to use the other isomer unless it were assumed that all organisms are descended from a single common ancestor. Since there are objections to both hypotheses of the biological origin of optical specificity perhaps neither is valid. No matter what the method, once some degree of optical specificity was achieved, this purity needed to be enhanced. It seems plausible to suppose that enrichment occurred by interaction of the directive effect of the small degree of asymmetry both as reactant and as catalyst and accompanied by interruptive processes. By these means specificities in reactant and catalyst could reinforce each other thus providing a higher order of specificity in the products if the reaction is stopped before reaching equilibrium. Each consecutive step should increase the optical purity still further in the manner outlined in the discussion of the maintenance of optical purity.

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## CHAPTER XIV

# Chemical Disinfection

### DEFINITIONS

The poisoning of bacteria by chemical substances has been of great interest to both the practical-minded and curious-minded investigator. The insight into bacterial metabolism which would be provided by a true understanding of the mechanisms of chemical poisoning and its practical implications has stimulated a tremendous volume of activity. Much information has been accumulated, and numerous opportunities have been afforded for the development of theoretical concepts of the nature of chemical disinfection.

In studying the chemical poisoning of bacteria two choices are available. The poison may be added to a population and the effects on metabolic function or survival studied or additions may be made to developing cultures and the effects on growth rate and crop yield noted. A loss of viability as measured by the inability of the organisms to grow and multiply when transferred to a fresh medium is referred to as a *bactericidal*, *disinfectant*, or *germicide* effect. An action resulting in the temporary loss of reproductive capacity or a large decrease in reproductive rate is said to be *bacteriostatic*. Since the best objective test of loss of viability of bacteria is their ability to multiply when transferred to a suitable nutrient environment, *death* has been defined by the bacteriologist as the permanent loss of the ability to multiply. In this sense death is synonymous with *sterile*, the term generally employed by biologists to signify the loss of power to reproduce.

In medical parlance the appellation disinfectant is commonly limited to those chemical agents applied to inanimate objects for the purpose of killing bacteria or other microbes. Bacteriostatic substances applied to living tissues are known as *antiseptics*. A *chemotherapeutic agent* is one which *in vivo* effectively limits bacterial growth or is bactericidal at concentrations which are non-toxic for the parasitized host. The term disinfectant is not used to describe chemotherapeutic agents. Nor are those chemotherapeutic agents used parenterally called antiseptics. On the other hand, many chemotherapeutic substances are employed topically, the use to which antiseptics are limited. It may be purposeful to have different words in order to classify poisons according to the varying uses poisons are put to by sanitary engineers and physicians. Nonetheless, such a classification does not necessarily separate poisons according to their mode of activity. It has been said that



chemotherapeutic agents act on bacteria by interference with specific metabolic functions in contrast to antiseptics and disinfectants which act as gross protoplasmic poisons. This distinction is more artificial than real. The sulfonamides, which are chemotherapeutic agents, can be shown to have the capacity to inhibit numerous and diverse biological activities and in high enough concentrations can act as bactericides, though their therapeutic usage is limited commonly to bacteriostatic concentrations. On the other hand mercuric chloride, a disinfectant, can act as a bacteriostatic agent under proper conditions, and chlorine, a disinfectant in everyday use in tremendous quantities, has been claimed to act by specifically inhibiting particular enzymes involved in glucose oxidation.

If exposed bacteria are washed free of the solution containing a poison and are transferred to a nutrient medium, growth may or may not ensue depending on the nature of the washing solution and procedure. Such observations indicate reversibility of a poisoning mechanism. (Frequently the word *narcosis* is used to refer to any such reversible inhibition of a biological process by a chemical substance normally extraneous to the natural system and *narcotic* to the substance causing the inhibition.) If the composition of nutrient media into which poisoned organisms are seeded is varied, differences in the ability of the culture to grow out may be noted. Observations of these kinds emphasize that the bactericidal action of a poison is not an absolute phenomenon but rather is relative to the treatment organisms receive after exposure to the poison.

It is also known that varying the concentration of a poison will result in a graded response ranging from a minimum effect on growth rate at low concentrations to an inability of the organism to multiply under any circumstances when exposed to high concentrations of a poison. The gradual merging of a bacteriostatic effect into a bactericidal one has been interpreted to mean that the mechanisms of the two actions are similar and differ only in a quantitative way.

This interpretation has not gone unchallenged. Van Esseltine and Rahn (1949) claim that several kinds of observations cannot be reconciled with the assumption that with a given poisonous substance, bacteriostatic and bactericidal mechanisms are qualitatively similar and vary only in degree. As an example it is pointed out that crystal violet is more bactericidal against young than old cultures of bacteria, while bacteriostatic effects are greater with old cultures. Variations in pH in these cases affect the bacteriostatic efficiency of the dye relatively more than they do the bactericidal efficiency.

These observations while suggesting the existence of separate mechanisms for bactericidal and bacteriostatic actions do not prove this to be true. Since the basophilic character and permeability of bacteria in young cul-

will probably be forthcoming eventually. The following possibilities deserve consideration insofar as they can be shown to apply to particular cases.

Inevitably an added inhibitor first comes into contact with the surface structures of organisms. When only small quantities are added the poison may be concentrated at the surface and thus not pass in any significant amounts into the interior. As such, permeability changes may ensue which could result in a freer flow of metabolites across the membranes and an increase in metabolic activity when the permeability properties have been rate limiting in the normal activities of the organism. Probably there is always some permissive variation in the permeability properties of organisms which in itself is not harmful until exceeded.

The principle for which the case of permeability provides an example may be stated in a more general form; namely, that in any series of dependent processes a quantitative decrease in the influence of an inhibitory or limiting process will have the result of increasing the activity of the other members of the series. This can happen when the threshold dose for poisoning an inhibiting or limiting process is lower than for the dependent processes.

If two processes compete for a single substrate, then in the presence of limiting quantities of substrate the inhibition of one process will result in an increase in the rate of the competing process. This can occur when a poison is specific in its action and affects only one of the competing systems. As one example of a fairly well understood case, the stimulation by cyanide of bacterial luminescence at limiting concentrations of oxygen may be mentioned. The luciferase-luciferin system of the bacteria is insensitive to cyanide, but the cytochrome system of the respiratory mechanism of these aerobes is sensitive. As a result the addition of cyanide prevents transfer of hydrogen to oxygen by the respiratory mechanisms with a consequent increase in the amount of oxygen available for participation in the luminescent system.

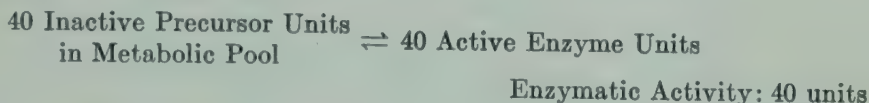
Stimulation may also occur with a non-specific poison capable of inhibiting all of the members of competing processes. The only requirements are that the threshold concentrations for poisoning the individual systems be dissimilar and that the concentrations of poison employed be low enough so as not to exceed the minimum concentration required to inhibit the less sensitive competitive processes.

If more than one product can result from the reaction of an enzyme with a poison and if one or more of these products remain catalytically active then addition of small amounts of the inhibitor may actually cause an increase in the total amount of active enzyme present in a biological system. A hypothetical case is illustrated in Figure 92.

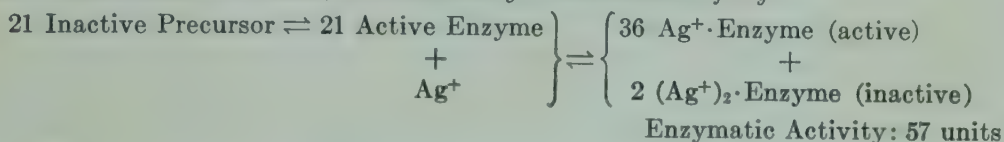
Bacterial multiplication, assimilation and catabolism are sensitive in

decreasing order to *indifferent inhibitors*, narcotic substances which are non-specific in the biological processes they act upon. Consequently, at low concentrations of these inhibitors it is not unusual to have an inhibition of assimilation with an accompanying increase in catabolic processes (Table 51). In these cases it is quite probable that there is competition for common metabolites by various assimilatory and catabolic processes. The poisoning of the more sensitive anabolic reactions makes more of these metabolites available for catabolic reactions. In Fig. 93 a hypothetical mechanism of metabolism is illustrated to show how such situations might occur.

*Normal State in Absence of Added Ag<sup>+</sup>*



*Low Concentration (41 moles) or Stimulatory Concentration of Ag<sup>+</sup>*



*High and Inhibitory Concentration of Ag<sup>+</sup> (101 moles)*

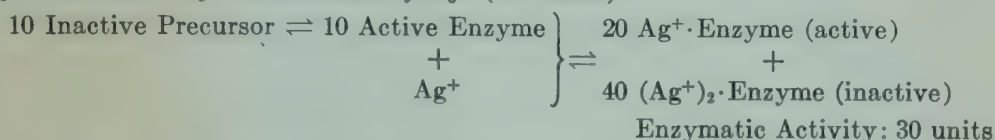


FIG. 92. A hypothetical case of stimulation based on the assumption that a number of reaction products result from the combination of a poison and enzyme, not all of which are incapable of enzymatic activity. In the case illustrated the amount of biological material is held constant and the concentration of the poison (silver cation) is varied.

## QUANTITATIVE STUDIES OF DISINFECTION

### GENERAL CONSIDERATIONS

Comparisons of the quantitative effects of different factors upon the disinfection process are made by observation of the death rates within populations of bacteria exposed to a toxic chemical under varying conditions. Similarly the relative toxicity of different compounds may be evaluated. Theoretically these studies of the dynamics of the bactericidal process should also assist in understanding the mechanisms of action of poisons. In actual practice different and mutually exclusive interpretations of mechanisms of action have been deduced because the customary methods of plotting data display no regularity in the trend of the death rate with time in populations of bacteria exposed to varying concentrations of a poison.



Since it is simpler to determine the number of survivors rather than of dead organisms in an exposed bacterial population, disinfection curves traditionally have been constructed by plotting the number of survivors or the logarithm of the number of survivors against the time of exposure. Three types of curves have been obtained: sigmoid, exponential, and exponential preceded by a lag phase. The occurrence of exponential curves

TABLE 51

*A list of a few agents which affect the coupling of oxidation-reduction reactions with energy utilization*

INHIBITOR	ORGANISM OR TISSUE	PROCESS STIMULATED	PROCESS INHIBITED
Phenyl urethane	Sea urchin eggs	Respiration	Cell division
Chloral hydrate or chlorotone	Luminous bacteria	Respiration	Luminescence and assimilation
Phenobarbital and amytal	Luminous bacteria	Respiration	Luminescence
Various substituted phenols	Sea urchin eggs	Respiration	Cell division
Dinitrophenols	Frog muscle	Respiration and glycolysis	Maintenance of phosphocreatine
Dinitrophenols	Root nodule bac- teria	Respiration	Assimilation and glucose uptake
Dinitrophenols	Sperm	Respiration and glycolysis	Motility
Dinitrophenols	Yeast	Respiration and glycolysis	Assimilation
Dinitrophenols	Yeast	Respiration and glycolysis	Assimilation and phosphate uptake
Azide	Yeast	Fermentation	Assimilation
Azide	Frog muscle	Glycolysis and res- piration	Oxidation of lactate
Gramicidin	<i>Staphylococcus aureus</i>	Oxidation	Assimilation and phosphate uptake

(From McElroy, 1947.)

has been sufficiently frequent to lead some bacteriologists to the conclusion that this type of curve represents the typical case and is proof of a monomolecular reaction as the cause of death.

This kind of reasoning from the shape of a survivor-time curve to the nature of the reaction of poisoning has been extended to cases of non-exponential curves which often bear a resemblance to those expected if the cause of death were a bi-, tri-, or other multimolecular reaction. This reasoning is tenuous as in truth a number of different equations can be fitted equally well to a given body of disinfection data. The precision of the

methods employed for estimating the numbers of survivors is not sufficiently refined to permit the choice of one equation as best from among the number of possible applicable equations.

As discussed previously, a hypothesis of the monomolecular order of death rests on the assumptions that all organisms in the bacterial population are of equal resistance and that the death of the individual organism is due

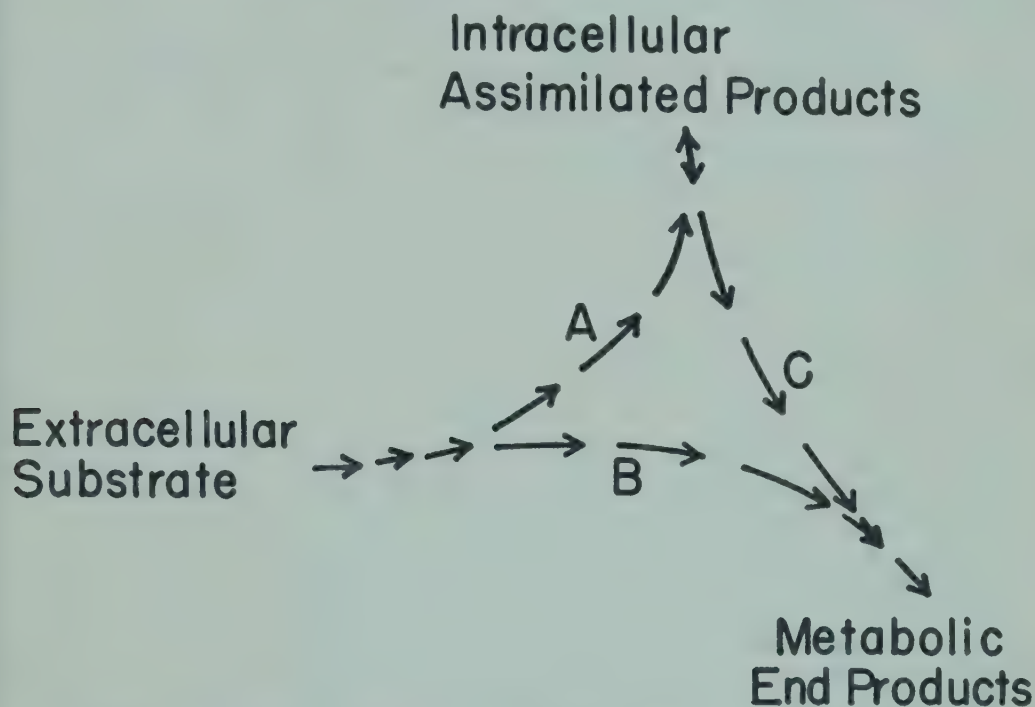


FIG. 93. A schematic diagram of metabolism. Low concentrations of narcotics preferentially inhibit assimilation and thus would interfere with the sites of reactions indicated by A. When A is blocked the intermediates in that portion of the pathway operating in common with series B become available for the exclusive utilization of the reactions of B. In this way the catabolic pathway B may actually increase in rate in the presence of a narcotic. The catabolic endogenous system (C) may or may not be affected, depending on whether the inhibitor acts on a step common to both processes A and C.

to the reaction of a single molecule. From knowledge of the effects of poisons on other organisms and of the essential similarity of different kinds of protoplasm it would not seem likely that bacterial populations would possess uniquely uniform resistance to poisons. Even for the case of a clone it is improbable that at any given moment all the individuals will be exactly alike since variations in different areas of the culture medium might be reflected in differences among the individuals growing in the medium. Furthermore, the bacteria exposed to a poison would not all be at identical stages in their cycle of fission, a fact which would be reflected by numerous

differences among the individuals, as for example, their body size and weight and the stage of synthesis of transverse septa. The former factors are generally known to be important variables affecting the resistance of non-bacterial organisms to poisons and probably are equally important for bacteria. It is not unreasonable to suppose that differences in size and shape are influential by causing variations in the time taken by disinfectant molecules to diffuse from the surface to reaction sites within the organisms.

If in actuality the natural variations in resistance within a bacterial population are definable by some probability curve, the application of very potent concentrations of the poison might obscure this fact since the differences among the more susceptible elements of a population dying at a very rapid rate would be experimentally indistinguishable with any degree of accuracy. In such a situation the determination of the number of survivors at the end of the first experimental interval of time of exposure to the poison would not give any information as to the variations in the resistance that had existed among the organisms dying within this period. Instead they are lumped together as though they were a homogeneous group. Mathematically it can be shown that this behavior can result in an apparently skewed distribution of resistances and in a spurious curve for survivor time similar in shape to an exponential curve. That this situation actually occurs is demonstrated by the data plotted in Figure 94. The opponents of the monomolecular theory of death have therefore stressed that a plot of the numbers of survivors after time of exposure can only have significance for theoretical studies of the dynamics of disinfection when conditions are employed which permit the observation of any differences in resistance existing within the more susceptible half of the population. The great bulk of the population should not die within the period of the first one or two experimental observations.

A further argument adduced against the use of an exponential survivor-time curve as proof of the hypothesis of a monomolecular order of death caused by chemical poisons is that graphical representation of certain naturally existing skew distributions of resistance would resemble exponential curves.

For many years biologists in diverse fields of study have known of the existence of skew distributions of the variations of biological characteristics. In these cases a more nearly normal probability curve is obtained when the logarithms rather than the arithmetic values of the measurements of the biological character are plotted. Repeatedly, pharmacologists have made similar observations in studies of the effects of drugs on animals and plants. These findings are not surprising when it is recalled that for natural phenomena the interrelationships of dependent variables are more often expressed by some exponential function rather than by a coefficient of the



variable. The significance of these considerations lies in the suggestion that biological characters will frequently vary in proportion to some exponential function of the weight, size or other characteristic of the organism. Gaddum

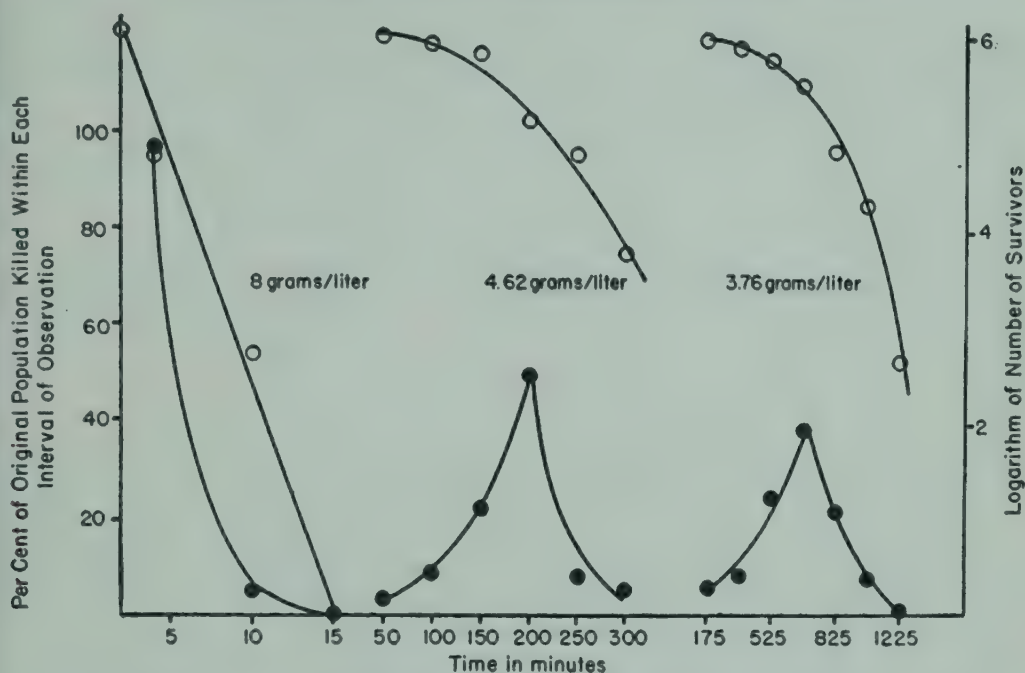


FIG. 94. How an apparently skew distribution in resistance of a bacterial population (*Escherichia coli*) may lead to a logarithmic survivor curve simulating that of a monomolecular order of death. Reading from left to right the bottom curves (—●—) are distribution curves of the resistance manifested toward three concentrations of phenol of increasing potency. When these same data are plotted (—○—) in the traditional manner as the logarithms of the number of survivors after varying periods of exposure (a cumulative frequency curve) the most potent concentration of phenol which kills the bulk of the population rapidly and within the first period of observation yields an exponential curve, whereas lesser concentrations do not. Thus the exponential curve in this case cannot be interpreted as meaning that the population is composed of organisms of uniform resistance, but does imply that the technique for estimating survivors with a rapidly acting poison is inadequate for distinguishing differences in resistance within the population. An alternate but unacceptable explanation would be that the highest concentration of phenol has a different mode of action than do the lesser concentrations.

(Data taken from Jordan and Jacobs, 1944a)

illustrates the point by reminding us that the growth response of an elephant and of a maggot to a hot summer day is in proportion to some power of the size rather than the actual size of the animals. The elephant grows and adds ten pounds to its weight while the maggot which proportionately grows more rapidly, adds only a hundredth of a milligram to its body substance (see Fig. 95).

It has been found for a large body of data obtained from investigations of different bacteria and disinfectants that a normal distribution in the resistance of individuals in bacterial populations is shown if numbers or per cent of survivors is plotted against the *logarithms* of the times of exposure. Disinfection data which yield different kinds of curves by other methods of plotting do show a more nearly normal type of distribution

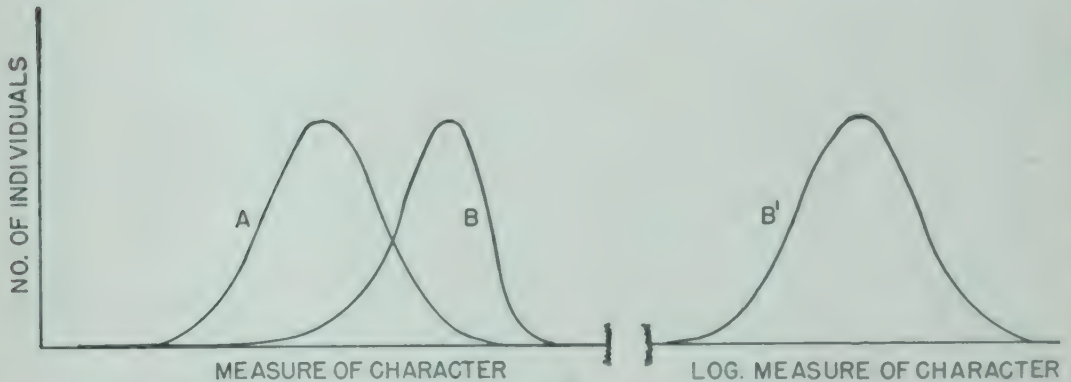


FIG. 95. Shapes of distribution curves of a characteristic that varies within a population as an exponential function of some other character which is itself normally distributed within the population.

Within any bacterial population composed of individuals of different weight or size which are normally distributed (curve A), any characteristic, such as resistance to a poison, dependent on body weight might well vary as some exponential function of the weight of the individual organism. Thus, the distribution curve for the dependent character would appear to be skew (curve B). In curve B the dependent character was assumed to vary as the cube root of the weight ( $W$ ) of the individuals, that is, as  $W^{1/3}$ . In actual fact biological characters dependent on body size may vary as exponential functions of weight greater than 0.33. Distribution curves would therefore be less skewed than curve B when the arithmetic value of these dependent characters are plotted. On the other hand, if the logarithm of the measurements of the dependent character are plotted, more nearly normal distribution curves are obtained as is illustrated by curve B'. Curve B' is a plot of the same data used to obtain curve B except that logarithmic values of the measurement of the dependent character have been plotted.

curve when the logarithmic values of exposure times are plotted. Furthermore, the same normal distribution of the logarithms of the quantitative measures of a characteristic within a population is common to numerous biological systems. These latter two facts seem to resolve in the affirmative the vexing question of whether bacteria act like other organisms in their behavior toward poisons and exhibit a varied capacity for resistance.

When dealing with normal distributions, data may be conveniently studied by plotting on *probability* graph paper. If the resistance within a population is normally distributed, the percentage of survivors plotted

against time on this graph paper will yield a straight line. The use of probability paper has an advantage in that the slope of the straight line is the reciprocal of the standard deviation of the survival times and thus readily provides an estimate of the extent of variation in resistance within a population. Nonetheless in itself the straight line cannot be taken as proof of the existence of variation in resistance because as mentioned before the precision of the data of disinfection permits several equations to fit the data equally well.

If the logarithmic rather than the arithmetic values of resistance are normally distributed, this fact will be revealed by the occurrence of a straight line only when per cent survivors against time is plotted on *logarithmic probability* graph paper. With this paper one is plotting logarithmic values of time.

#### THE CONCENTRATION EXPONENT

In order to evaluate the relationship existing between the concentration and killing effect of a disinfectant an appropriate range of concentrations must be studied. An empirically derived equation expressing the relation is  $C^n t = k$  where  $C$  is the concentration of disinfectant,  $t$  the time necessary for killing, and  $n$  and  $k$  are constants. On converting to logarithms,

$$\log t = -n \log C + \log k,$$

thus values of  $\log t$  plotted against  $\log C$  yield a straight line,  $n$  being the slope of the line and  $k$  the intercept. The slope  $n$  represents the factor expressing the change in disinfectant action with changes in concentration, and is known as the *concentration exponent* or the *coefficient of dilution*. It is obvious that the effects of dilution on the speed of killing will be greater for disinfectants possessing the larger  $n$  values. For disinfectants having the same coefficient of dilution, a difference in the constants  $k$  would represent the difference in time required for killing which is a measure of the relative potency of the substances, a smaller  $k$  value representing greater potency. Since most disinfectants do not have the same coefficient of dilution,  $k$  has little practical use.

While for any given set of conditions the coefficient of dilution of a disinfectant is characteristic, it is quite probable that the value will change in the presence of other chemical substances in the environment. Unfortunately, few specific data have been collected on this point. With temperatures near or below 20°C. there is reported to be only a slight variation in  $n$ , however, at higher temperatures  $n$  diminishes indicating a lessened effect of dilution upon disinfectant action. These observations do not favor one of the possible theoretical interpretations of the significance of  $n$ , namely that  $n$  represents the number of disinfectant molecules required for



reaction with receptor molecules or sites within the organism. This number should be independent of temperature and the presence of extraneous substances if the mechanism of disinfection is independent of the concentration of the drug. The lessened importance of dilution at temperatures above 30°C. could be due to an additive effect on the poisoning by the independent heat denaturation of protein.

With some substances a change in  $n$  is noted at different concentrations. Such a finding does not necessarily signify that the above equation is incorrect. It may indicate that different conditions or even mechanisms of death are coming into play at varying concentrations.

It is not the concentration of a poison added to a bacterial suspension but the *effective concentration*, the amount accumulating at the bacterial surface and within the organism, which is responsible for toxic activity. Therefore, in evaluating the influence of concentration it must be remembered that the organisms and the suspending medium are a system of discontinuous phases. Any factor increasing the accumulation of a poison at the interface between the bacteria and the suspending medium may increase the potency of a given concentration of poison. At the least the rate of transfer of a toxic material across the cell boundary will be affected by the tendency of the substance to accumulate at interfaces. It is probable that changes in  $n$  under different conditions are often linked to this fact. In other words, changes in conditions affect the effective concentration of the drug and not the number of molecules required to harm the bacterium.

It is also important that under a fixed set of conditions surface tension is not proportional to concentration of substances over the entire range of solubility. If it is permissible to consider surface tension measurements on solutions of a poison as a valid index of the tendency of the poison to accumulate at the bacterial surface then it is not surprising to find that  $n$  may be constant only for a limited range of concentration of a bactericide. Aggregation of solute molecules or micelle formation may also lead to changes in  $n$  since both the permeability and sensitivity of a bacterium may not be the same toward individual molecules and clusters of molecules of a poison. Figure 96 is an example of the correlation obtained between the change in  $n$  with changes in surface tension of solutions of varying concentration of disinfectant. Note how the changes in the slope of the disinfection curves parallel the changes in surface tension of the solutions of disinfectant.

From these considerations it is clear how the addition of extraneous materials may affect the concentration exponent of a disinfectant. In a system of discontinuous phases if the solubility of a poison is changed, its potency will be affected. The lowering of the solubility of a poison in water acts to increase the poison's concentration at the bacterial surface which generally tends to be less hydrophilic than the solvent system, and therefore

to increase the rate of any reaction taking place at the interface at the bacterial surface. In other words, even though the concentration of the disinfectant in the total system is fixed, the addition of any factor tending to decrease the solubility of the disinfectant is equivalent to an increase in concentration while addition of factors increasing the solubility is equivalent to a decrease in concentration of disinfectant. The principle is illustrated in Table 52.

One practical consequence of this knowledge is the indication that colloidal preparations of chemicals such as emulsoids or ointments may be

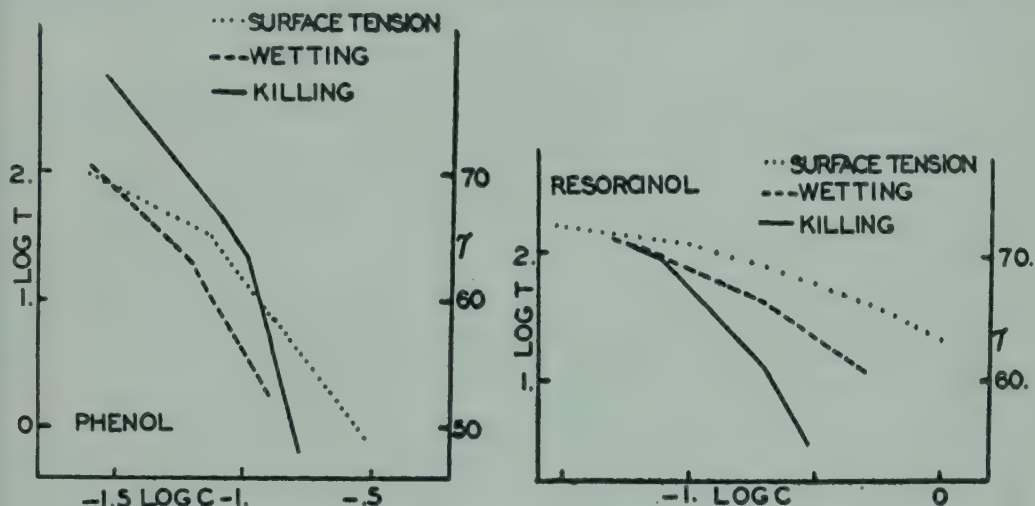


FIG. 96. The effect of change in the concentrations of phenol and resorcinol upon surface tension and upon the time for killing staphylococci and for wetting wool.

(From Cowles, 1940)

The implications of these data are two-fold; namely, that the effective concentration of a disinfectant is not the actual concentration added to the system, and that surface tension and wetting of a hydrophobe may be employed as indirect measures of the effective concentration.

preferable to solutions in certain practical uses for germicides. If bacteria are brought into the interfaces of colloidal systems they are exposed to the higher concentrations of poisons at the interface. Direct microscopic studies have shown that with emulsoids such as cresol preparations the suspended particles of the disinfectant may actually be readily adsorbed to the bacterial surface. Thus, the actual concentration of the disinfectant in immediate contact with the bacteria is much higher than would be estimated from the concentration of the disinfectant added to the total system.

#### *Effect of the Level of Mortality on the Concentration Exponent*

In calculating the coefficient of dilution an endpoint for the action of the disinfectant must be chosen. Practical interest in disinfectants has centered attention on their ability to sterilize so that the time it takes to rid a culture



of all viable bacteria has been generally employed as the endpoint. Unfortunately, the endpoint for estimating time of sterilization is arbitrary: when survivors are present in small numbers, aliquots of the culture taken for detection of viable organisms by plating or by other methods may not include viable organisms by chance alone. With prolonged exposure the still viable but increasingly injured bacteria may be more difficult to detect since they can become more demanding in their growth requirements. Moreover, the endpoint varies with the length of the incubation time employed for cultivation of survivors. Also some few unusually resistant variants may survive long after the remainder of the population has succumbed.

TABLE 52

*The effect of alcohol on the solubility of mercuric chloride and upon the toxicity of this compound towards anthrax spores*

	ETHYL ALCOHOL, PER CENT BY WEIGHT									
	0	5	10	15	20	25	30	40	50	51
Solubility of HgCl <sub>2</sub> (grams/100 ml).	6.24		5.63	5.43	5.15	5.08	5.86	7.58		11.3
Colonies Develop- ing*	113	383	199	184	86	70	287	335	1824	

\* The toxicity of 1.75 grams of HgCl<sub>2</sub> per 100 ml of the indicated alcohol solution was tested by noting the number of colonies developing from an inoculum with a fixed number of spores. With this fixed concentration of HgCl<sub>2</sub> the 25 per cent alcoholic solution was the most bactericidal one. The HgCl<sub>2</sub> also proved to be least soluble in this solution of alcohol.

(From Laird, 1920; after Paul and Krönig, 1896.)

Possibly this last factor may explain the often observed sharp breaks yielding a reduced slope in survivor curves after the major portion of the population has become non-viable. For these reasons Withell (1942) has suggested that it would be more satisfactory to determine the time required to kill 50 per cent of the bacteria (LT<sub>50</sub>), an endpoint whose superiority over the 100 per cent endpoint is attested to by its general adoption by biologists studying toxic substances in other organisms. With any given concentration of a poison the LT<sub>50</sub> or any other statement of the time it takes to kill a fixed percentage of the exposed population may be obtained by interpolation from a survivor-time curve plotted on logarithmic probability paper.

While the choice of the LT<sub>50</sub> as a more reproducible endpoint of disinfection than sterilization time (LT<sub>100</sub>) may be valid, it has been found that the LT<sub>50</sub> is not necessarily the best choice of endpoint. In Figure 97 data are recorded which show that with phenol the concentration exponent varies



with the percent mortality chosen as the endpoint for measurement of disinfectant action, and that this variation exists for the  $LT_{50}$  determinations when it is absent at higher levels of mortality. It is not known whether or not the influence of the level of mortality is itself subject to the nature of the disinfectant and to the kind of bacteria studied.

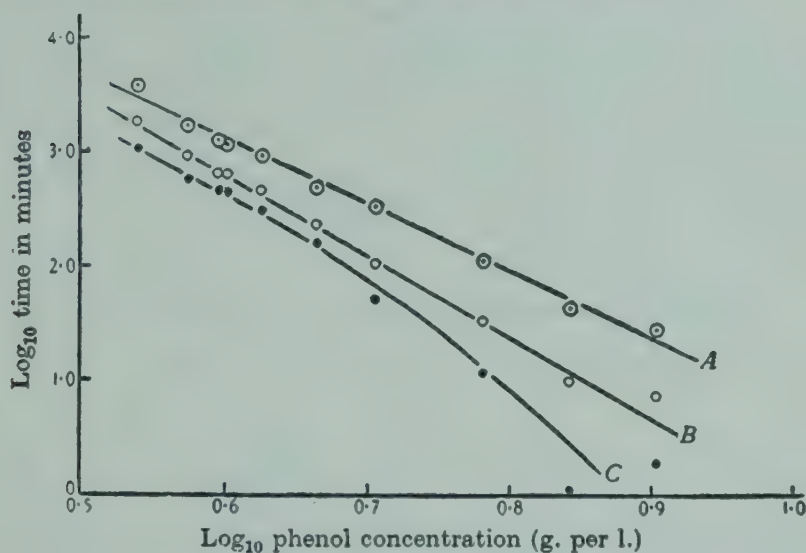


FIG. 97. The relationship between the logarithms of the phenol concentration and time necessary to achieve particular levels of mortality. Curve A represents the logarithm of the time required to reach an endpoint of 99.99999% mortality, curve B 90% mortality, and curve C 50% mortality (the  $LT_{50}$ ). The slopes of the curves which represent the coefficients of dilution are not parallel nor even constant in the case of the  $LT_{50}$ . These data demonstrate the dependence of the coefficient of dilution upon the level of mortality chosen as the endpoint. When the  $LT_{50}$  is employed as the endpoint level of mortality, the coefficient of dilution is shown to vary with the concentration of phenol.

(From Jordan and Jacobs, 1944)

#### *Thermodynamic Comparison of the Effectiveness of Narcotics*

A considerable amount of effort has been expended on methods of correlating the biological activities of various classes of drugs with some chemical or physical property exhibited by such compounds. A measure of success has been achieved in the case of substances known to act upon particular metabolic reactions. These specific metabolic antagonists owe their activities to their chemical structures as in the inhibition of succinic dehydrogenase by malonate discussed earlier.

On the other hand those inhibitory substances whose actions are more general have not been cataloged readily using classifications based on chemical structure. The data of Table 53 reveal that the members of the homologous series of normal primary alcohols differ greatly in their ability to

inhibit the emission of light by luminescent bacteria. Inasmuch as the molar concentrations of the alcohols shown differ by a factor of  $10^4$ , it is clear that structure alone cannot be the decisive factor in the narcotic effect.

As a solution to the problem of establishing a common basis for comparing substances, the use of thermodynamic activities of poisons has been proposed. The result of this basis of comparison is shown in the last column of Table 53. Here it is evident that similar thermodynamic activities will produce a given narcotic effect. Studies in various animal systems show that the correlation is not limited to the alcohols listed but includes secondary and tertiary alcohols, chloroform, acetone, methyl acetate and the like. Therefore the use of such a basis has some general merit.

TABLE 53  
*Suppression of luminescence in bacteria by alcohols*

NORMAL, PRIMARY ALCOHOLS	NARCOTIC CONCENTRATION	$A (\times 10^3)$
	<i>mols/liter</i>	
Methyl.....	2.76	8.0
Ethyl.....	0.90	6.3
Propyl.....	0.25	6.6
Butyl.....	0.076	7.4
Amyl.....	0.019	7.3
Hexyl.....	0.0056	9.0
Heptyl.....	0.00092	6.1
Octyl.....	0.00022	4.9

(From Brink and Posternak, 1948.)

However, some narcotics do not obey this relationship in all cases. Diethyl ether, for example, does not always function at the thermodynamic activity exhibited by other substances and is a typical exception in some biological systems but not in all. Furthermore, certain biological test systems result in scattered data for otherwise consistent compounds, apparently due to peculiarities of the test systems themselves. Thus complete generalization of the relative efficacy of narcotics is not possible at present. It may be that special mechanisms are operating in those cases which seem to be exceptional. Perhaps there are special problems of penetration, special mechanisms of detoxication, and the like which prevent equilibrium.

It will be recalled from an earlier section that the thermodynamic activity of a substance may be regarded as the effective concentration of that substance. When dealing with the dilute solutions of about 0.01 molar or less as is customary with narcotics, the thermodynamic activity may be calculated using laws of ideal solutions. If the narcotic has a relatively high vapor pressure and if the pressure of the narcotic in the vapor phase in

equilibrium with the narcotized cell can be measured, then the thermodynamic activity at equilibrium in the narcotized cell is:

$$A = P/P_0 \quad (1)$$

where  $A$  is activity,  $P$  the partial pressure of the narcotic above the system, and  $P_0$  the vapor pressure of the pure narcotic.

Activity coefficients ( $f$ ) are factors which yield the thermodynamic activities when multiplied by the concentrations. Thus if values of the coefficients are available the activities themselves are readily obtained from

$$A = fN \quad (2)$$

where  $N$  is concentration expressed as mole fraction. The mole fraction of a solute is the number of moles of solute divided by the number of moles of all substances present. For the dilute solutions of concern here

$$N = \frac{\text{moles of solute}}{\text{moles of water}} \quad (3)$$

Equation (2) is quite useful since values of  $f$  are available in the literature for many common narcotics.

If the drug is soluble to such a limited extent that a saturated solution is still quite dilute, then the thermodynamic activity can be obtained from still another relationship:

$$A = N/N_2 \quad (4)$$

$A$  and  $N$  have their previous meanings and  $N_2$  is the mole fraction of narcotic in a saturated solution. Since both  $N$  and  $N_2$  are easily measured, equation (4) is applicable to the slightly soluble substances.

The use of thermodynamic activities for comparing the effectiveness of narcotics has much to recommend it in the simplicity of the calculations and in the comparatively general scope of the results. The method is a valuable one in spite of the exceptional situations mentioned earlier.

#### COMPARISON OF DISINFECTANTS

Numerous methods have been proposed for the estimation of the prophylactic or therapeutic value of germicidal substances. In view of the many environmental factors which influence the bactericidal activity of chemical substances, the diversity of conditions and organisms against which they are directed in everyday practice and the differences in response among these substances to the same variables, it is probable that the search for a single test, numerical value, or coefficient (i.e., *phenol coefficient*) to express the relative worth of disinfectants will remain a will-o'-the-wisp as a scientific venture.



For scientific purposes a comparison of disinfectants under a *given set of environmental conditions* may be made by calculation of their *true reaction velocities* of disinfection,  $K$ , in the equation,

$$KC^n t = \log \frac{B}{b}$$

in which  $C$  is concentration,  $n$  the coefficient of dilution,  $t$  a time of disinfection preferably long enough to kill fifty per cent or more of  $B$  the initial number of organisms, and  $b$  the final number of organisms. The best estimate of  $K$  would require that for the determinations of the values of  $n$  a similar level of mortality be chosen for both disinfectants and that  $n$  be independent of the level of mortality chosen. These considerations should emphasize that scientific comparisons of disinfectants are not to be made without a considerable expenditure of effort.

## FACTORS INFLUENCING DISINFECTION

### BIOLOGICAL FACTORS

The harmfulness of poisons varies with the nature of the species to which they are applied, the outstanding example being the large number of such differences which are known to be correlated with the gram reaction. Irrespective of whether susceptibility varies among species of similar or dissimilar gram reactions, ultimately all differences in species resistance must be traceable to basic differences in the structural organization and metabolic processes of bacteria. Some of these causal factors may be mentioned briefly.

The known differences in assimilatory mechanisms are reflections of the varying composition in the complement of enzymes in bacteria. Thus bacteria must inevitably exhibit differences in their susceptibility to specific enzyme poisons. Variations in the capacity to produce neutralizing substances or antidotes, in the existence of enzymatic mechanisms of detoxification, in the ability to utilize alternative metabolic pathways for blocked processes, and in the acid-base dissociation constants of species-specific proteins and thus the capacity for formation of salt-like linkages with ionic disinfectants all contribute toward explanations of how the nature of the species affects susceptibility to disinfection.

Biological characters which influence the rate of diffusion of disinfectants to the sites of action must also be considered to play a role in species susceptibility. Prominent among these properties are differences in permeability which would act as limiting factors in the cases of poisons acting on cellular components located within the interior of the organism. The tendency of a species to grow in clusters would have the effect of reducing the rate of diffusion of toxic substances to organisms buried within the mass.

Diffusion rates are also decreased by increases in the viscosity of suspending fluids, a factor to which the capacity of bacteria to produce and accumulate slime layer material would make an important contribution. The production of slime layer material capable of reacting with a disinfectant would have the further effect of reducing the effective concentration of the disinfectant.

### *Physiological Age*

Young actively multiplying bacterial cultures are less resistant to bactericides than are cultures beyond the logarithmic stage of growth. The

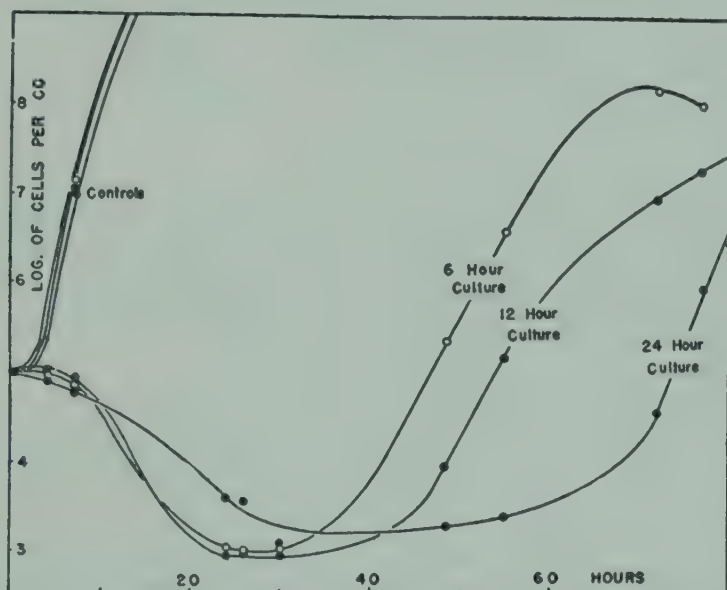


FIG. 98. Growth curves of *Escherichia coli* in nutrient media with 1:100,000 crystal violet solution starting with inocula of different ages.

(From Hoffmann and Rahn, 1944)

effect of the physiological age of a culture on bactericidal efficiency varies with the concentration of the disinfectant, being considerably more important at the higher concentrations. Thus a six hour culture of *Streptococcus lactis* showing no difference in resistance from 12 and 24 hour cultures to two parts per million of crystal violet has been noted to die off more rapidly than the older cultures when the concentration of the dye was increased five times.

With bacteriostatic concentrations of disinfectants it is often observed that the older bacteria are the less resistant types. Fig. 98 shows this relation for *Escherichia coli* with crystal violet. Bacteriostasis caused by crystal violet may be due to the tendency of this dye to poise the oxidation-reduction potential at levels unfavorable for the initiation of bacterial growth. Conceivably young organisms may be less susceptible to bacterio-

static levels of the dye, as they are more active than old bacilli in adjusting the oxidation-reduction potential of their environment.

*Escherichia coli* has also been found to be more resistant to the bacteriostatic action of sulfanilamide when the culture is actively multiplying. It is quite possible that any greater resistance of younger bacilli to sulfanilamide is attributable to their greater production of *p*-aminobenzoic acid, a normal metabolite synthesized by many bacteria which competitively inhibits the cellular uptake of sulfanilamide.

In summary it may be said that differences in susceptibility to disinfection based on the physiological age of organisms will be found to be caused by differences in chemical content such as lipids, nucleic acids and proteins, in concentrations of coenzymes and intermediate metabolites, and in permeability properties.

#### TEMPERATURE

Temperature increase has the effect of accelerating disinfection. Temperature coefficients are calculated from the velocity coefficients of disinfection at various temperatures, or more simply from the time required at various temperatures to bring aliquots of the same bacterial suspension to the same level of mortality.

Typical data on the temperature coefficient of disinfection are presented in Table 54 from which a number of conclusions may be drawn:

1. The temperature coefficient varies with the nature of the disinfectant and the species of bacteria.
2. The temperature coefficient for a disinfectant varies with the temperature. With a given species values close to one, to two, or higher, may be observed at different temperature ranges. It may increase or decrease with a rise in temperature, and it may show opposite trends with different organisms.
3. Extraordinarily high temperature coefficients can be observed.
4. The temperature coefficient of a disinfectant is not independent of the presence of extraneous substances and may vary greatly with changes in pH.

These findings emphasize the complexity of temperature effects on disinfection and the fact that temperature coefficient data do not serve for any simple exploration of the problem of mechanisms of poisoning.

When the logarithm of disinfection rate or mortality time is plotted against temperature, a straight line may result. More often, any linearity observed is limited to a narrow range of temperatures. Similar plots for each of a series of concentrations of a disinfectant may yield lines of similar slope, but more frequently the slope varies, tending to be less acute at the lowest effective concentrations. Based on data obtained from what appears



to be the most thoroughly controlled and precise series of experiments reported to date, Jordan and Jacobs (1946) have concluded that the true shape of temperature-logarithmic mortality time curves is sigmoidal in

TABLE 54  
*Temperature coefficients of disinfection*  
I. Disinfection by various alcohols

ESCHERICHIA TYPHOSUM				STAPHYLOCOCCUS AUREUS			
Disinfectant	10-20°	20-30°	30-40°	10-20°	20-30°	30-40°	40-50°
Phenol.....	5.8	8.3	8.4	5.1	3.9	4.0	7.4
Ortho-cresol.....	6.6	5.1	6.9	5.4	4.2	4.3	10.0
Para-cresol.....	6.1	5.8	5.6	4.3	4.1	4.4	9.0
Resorcinol.....	7.1	7.1	8.8	3.1	3.2	4.4	
Ethyl alcohol.....		43.0	54.0		13.0	9.0	
n-Butyl alcohol.....		31.0	40.0		11.0	9.0	

(From Tilley, 1942.)

II. Disinfection by chlorine at various acidities and temperatures  
(*Bacillus metiens* spores)

pH	25-36°	35-45°	45-55°
6.0	6.1	5.4	5.4
8.7	3.7	3.1	3.5

(From Charlton and Levine, 1937.)

III. Disinfection at varying pH with *Escherichia coli* as test organism

pH	0-10°	10-20°	20-30°
3.5	2.12	4.34	3.76
6.1	1.53	1.12	4.56
7.1	1.62	2.99	2.29
8.0	1.22	2.12	3.0

(From Cohen, 1922.)

character. They point out that the rate of disinfection becomes infinitely small if temperatures are lowered sufficiently. Thus a rise in temperature beyond this threshold or minimum temperature must be accompanied by a rise in the temperature coefficient. The complexity of the temperature effects on disinfection make it doubtful at the present stage of knowledge that sufficiently valid temperature coefficients could be calculated to serve for the characterization of disinfectants.

## ORGANIC MATTER

Generally the presence of organic matter will reduce the effectiveness of poisons. Since in the usual laboratory situation organic matter is of biological origin, it will include materials of the same chemical nature as exist in bacterial protoplasm, and will tend to reduce the effective concentration of poisons by competing with bacterial components in reacting with the poisons. An example is the protection against detergents afforded bacteria by phospholipids.

In practical situations biological matter will be present most frequently as particulate matter or in the colloidal state. Thus the effective concentration of surface active disinfectants could be reduced by an increase in the total area of extraneous solid surface-liquid interface at which accumulation will occur. This cause of the protection afforded by colloidal organic matter against chemical disinfection may be called a *protective colloid* effect. Actually the term protective colloid effect has been applied commonly to any example of protection by organic matter irrespective of its cause.

As in the case of protection of bacteria against heat it has been thought that increased resistance against chemical disinfection in the presence of organic matter might on occasion be due to a process of dehydration. Extensive hydration of organic matter could reduce the thermodynamic activity of water. Presumably such a mechanism would only be effective when disinfectants function by means of harmful hydrolysis reactions.

## ANTAGONISM AND SYNERGISM

The effect of simultaneous addition of two disinfectants to a system may be *additive*, namely, simply a summation of the toxicities expected from the compounds singly. When a range of concentrations and various ratios of mixtures are studied more complex phenomena often are observed. The compounds acting together may be more toxic than either acting alone, an example of *synergism*, or the inhibition may be reduced, an example of *antagonism*. Antagonism may extend to the observation of no toxic effects with mixtures of individually toxic quantities of the disinfectants (Fig. 91). The mixtures yielding antagonistic and synergistic effects will vary in concentration and the ratio of constituents depending on what biological function is studied, growth or particular chemical activities of the bacilli. The term antagonism is also extended to those cases of reduction in the inhibitory effect of a poison by the addition of non-toxic substances. Increased toxicity of a toxic preparation resulting from the addition of non-toxic substances may be called *synergism*; however, in the literature of pharmacology these cases have frequently been referred to as *potentiation*.

Examples of antagonism have been classified into three groups:

1. *Chemical* in which the antagonists react directly to form products of

reduced toxicity. The commonest example would be the neutralization of an acid by addition of a base.

2. *Physiological* in which case the two substances produce opposite effects by action on independent enzyme systems or cellular structures. A case of this sort is the antagonistic effect of the addition of coenzymes I and II on the inhibition of growth of *Staphylococcus aureus* caused by sulfapyridine. The antagonism is noted only when small inocula are used under which circumstance coenzymes I and II act as growth stimulating factors.

3. *Specific* in which one of the antagonists inhibits action of the other by competition for a common cellular substrate. In these cases there is no direct reaction between the antagonists.

In recent times much progress has been made in the detection of the enzymatic systems and cellular substrates involved in examples of antagonism in which direct reaction of the antagonists is not noted. It will, therefore, be appropriate to again consider the subject of antagonisms in the section dealing with the enzymatic basis of disinfection.

Much less progress has been made in understanding examples of synergism. A few of the possible causes of synergism may be listed:

1. The synergist may buffer or poise the environment at a more favorable pH or oxidation-reduction potential for the action of the toxic substance. This would apply to dissociable disinfectants which are only toxic in either the unionized or ionized state or to reversibly oxidized disinfectants toxic in only the oxidized or reduced form.

2. The synergist may increase the solubility or sorption of the disinfectant in a cellular component. This could result in an increase in toxic potency in two ways: the amount of the disinfectant in the cellular phase or component at which toxicity is expressed is increased; the amount of the disinfectant in cellular phases in which toxicity is not possible is decreased.

### MECHANISMS OF DISINFECTION

The reaction of a chemical poison with a cellular constituent may involve either a reaction of the oxidation-reduction, hydrolysis, salt formation, or metathetical types with some reactive group on a protein or other molecule or adsorption at an interface. The products of such a reaction or associated change in colloidal state may transform a metabolically active substance to an inactive state and otherwise interfere with the proper participation of metabolites in metabolic reaction chains. Actually, in living organisms it is only in a small proportion of the known cases of poisoning for which it is possible to state with a feeling of confidence the exact site and nature of the poisoning reaction.

While in a particular case it may be possible to determine the general nature of the reaction involved in poisoning, it commonly remains a difficult



task to identify particular cellular substrates which undergo the reaction or to be certain that only one kind of substrate is involved in the metabolic disturbance observed. Thus though there is no doubt that the mercuric ion poisons bacteria by reaction with sulfhydryl groups on protein molecules, it is not known whether the reaction which kills the organism is due to inactivation of one supremely important sulfhydryl enzyme or is the result of reaction with any number of different enzymes. The problem is further complicated by the fact that each toxic substance is observed to be capable of modifying a number of biological activities such as multiplication, glycolysis, oxygen consumption, and there are no known simple rules of procedure for distinguishing which of the inhibited functions is primary and which are the consequences of the primary disturbance.

The separation of fractions or the isolation of specific constituents of organisms for an *in vitro* study of their reactions with poisons, while logical as an attempt to evolve a simplified system for the study of mechanisms of disinfection, has been disappointing. In general no evident correlation is found in the concentrations of the poison required for the biological inhibition *in vivo* and the *in vitro* inhibition of cell-free enzyme preparations. A possible exception to this common finding is the observation with *Escherichia coli* that the bactericidal concentrations of chlorine are parallel to the concentrations required to inactivate the enzyme aldolase (Fig. 99).

Inasmuch as great difficulty has been experienced in discovering the exact mechanisms and sites of poisoning, it has been productive to consider the problem more broadly and to ask whether poisons act by affecting permeability, energy yielding or transferring metabolism, assimilation, or multiplication. The following sections are devoted to these questions.

#### DISINFECTION AND PERMEABILITY

Inasmuch as the bacterial surface functions in the maintenance of the integrity of the physico-chemical organization of the organism and probably acts as a metabolically active structure as well, a disturbance in the structure of the surface of bacteria is potentially the basic cause of bacteriostatic or bactericidal activity by chemical substances. Surface active toxic agents such as phenols, soaps, long chain aliphatic amines, amides, guanidines, and quaternary ammonium compounds probably owe their disinfecting properties to their ability to disrupt the permeability properties of the bacterial surface. Gramicidin and dinitrophenol have been shown to inhibit the transport of phosphate across the cell wall.

Actually there have been few attempts to demonstrate damage to the bacterial surface by direct cytological means. The study of the leakage of compounds from the interior of bacteria into the suspending medium has been a simpler and satisfactory, even if indirect, measure of a loss in

selective permeability properties. Direct chemical analysis for phosphorus and nitrogen escaping from the bacterial body has been employed, and in Table 55 it is shown that this criterion for cellular damage correlates well with the bactericidal activity of surface active agents. It should be noted that the ability of a surface active agent to act as a bactericide is not a

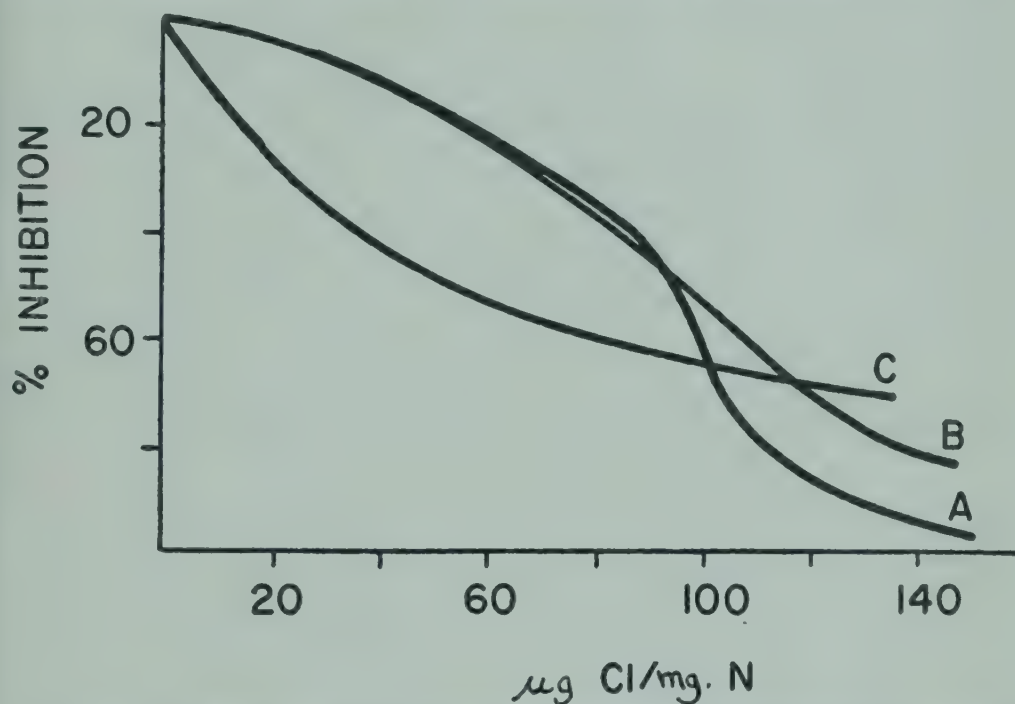


FIG. 99. The effect of chlorine on the viability (A), glucose oxidation (B), and aldolase (C) of *Escherichia coli*. The glucose oxidation was measured employing a resting cell suspension, while the aldolase employed was a cell-free extract of the enzyme. Note that the bactericidal effect of chlorine closely parallels the inhibition of glucose oxidation. The latter may be due to the inactivation of the enzyme aldolase which appears to be as sensitive to the lower concentration of chlorine as glucose oxidation and the viability of the bacilli. Unexplained remains the inability of the chlorine to completely inhibit aldolase activity *in vitro* at those higher concentrations which act on the other *in vivo* biological properties measured.

(Adapted from Knox, Stumpf, Green and Auerbach, 1948)

mere question of its ability to accumulate at the interface between the bacterium and the suspending medium. Additionally the agents must have the ability to combine with surface constituents if they are to be effective. Presumably it is this lack of combining power which is responsible for the inability of non-ionic detergents to cause bacteriostasis in comparison to the disinfecting power of the ionic surface active agents.

A substance need not be a typical surface active agent in order to disrupt permeability properties. Ionic copper, for example, capable of reacting with

TABLE 55

*Effect of surface active agents on staphylococci*

AGENT	CHEMICAL STRUCTURE	CONC.	KILLING EFFECT	PER CENT OF P AND N RELEASED†	
				P	N
		<i>µg/ml</i>			
Cationic agents:					
Triton K-60	Dodecyldimethylbenzyl ammonium	310	—	10	5
Zephiran	Alkyldimethylbenzyl ammonium	165	++++	80	70
Phemerol	<i>p</i> -Tertiaryoctyl-phenoxy-ethoxyethyl dimethylbenzyl ammonium chloride	500	++++	115	90
Ceepryn	Hexadecylpyridinium (pure)	500 125	++++ ++++	150 100	110 75
Tyrocidine	Basic lipid-soluble polypeptide	165	++++	105	130
Anionic agents:					
Duponol C	Lauryl sulfate	830	+++	100	65
Triton W-30	Alkyl phenoxyethyl sulfate	485	++++	110	105
Ultrawet A	Polyalkyl benzenesulfonate	500	—	1	15
Aerosol OT	Dioctyl sulfosuccinate	500	++++	145	105
Tricresol	<i>o,m,p</i> -Cresol	5000 1000 200	++++ — —	90 5 5	250 75 10
	<i>o</i> -Chlorophenol	5000 1000 200	++++ — —	100 10 5	90 0 0
ST-37	Hexylresorcinol	830 500	++++ ++++	95 105	125 110
Bile salt	Taurocholate	520	—	5	30
Non-ionic agents:					
Triton NE	Aryl alkylpolyether alcohol	520	—	15	4
Tween 20	Sorbitan monolaurate polyoxyalkylene derivative	500	—	5	50
Tween 80	Sorbitan monooleate polyoxyalkylene derivative	500	—	0	65
Arlacel C	Sorbitan monooleate (purified)	500*	—	0	75
Saponin	Steroid glucoside	500	—	0	5

\* Water dispersion of insoluble agent.

† Per cent of the amount extractable from normal bacilli with trichloroacetic acid. Values higher than 100 per cent are due to autolysis.

(From Hotchkiss, 1946.)



proteins but certainly not a substance ordinarily thought of as being a surface active agent can by direct methods be shown to change the permeability of red blood corpuscles for glycerol.

If a change in bacterial permeability is induced by a toxic agent, how can one know that any coinciding metabolic inhibition or bactericidal action shown by the substance is due to the change in permeability? Hotchkiss (1948) has suggested that if the action of a disinfectant is primarily on the bacterial surface then increasing concentrations of the disinfectant ought to reach a point beyond which there will be a plateau in the per cent inhibition of metabolic activity, and the inhibition will never be complete. The reasoning behind the adoption of such a criterion is the simple one that while disturbing the permeability will result in a decreased concentration of intracellular metabolites in proportion to the amount of poison added there cannot be a *complete* depletion of intracellular constituents by the mere process of dilution in the closed universe of a bacterial culture. Some finite quantity of metabolites will remain within the cells when the property of selective permeability is totally lost. Therefore, inhibition of metabolic activity cannot be 100 per cent effective when permeability is the sole biological function disturbed. This view is probably an oversimplification since threshold concentrations of certain reactants in the system may have to be exceeded in order to obtain any activity at all.

The ionic detergents are protein denaturants. Potentially they may interfere with the activity of any enzyme and for this reason in sufficient quantities may completely inhibit any metabolic activity and sterilize a culture. These observations do not invalidate the hypothesis of a primary effect by surface active agents on permeability. The fact is that the minimum concentrations of surface active agents which can cause bacteriostasis or kill bacteria are generally considerably lower than those concentrations required for detectable protein denaturation.

Fatty acids penetrate biological membranes as the unionized molecules. With bacteria it has been shown that the toxicity of the high molecular weight fatty acids is relatively independent of pH. The obvious interpretation of these findings is that these substances act at the surface and not within the organisms, otherwise one might expect a greater effect at those pH values where relatively less of the fatty acid is dissociated.

The classical lipid solubility theory of Overton has stressed the correlation often noted between the lipid solubility and potency of narcotics. The disinfecting activity of homologous series of compounds increases with the length of the carbon chain to a maximum and with further increase in chain length may or may not decrease depending on the series. Generally, the surface activity of the compounds as measured by surface tension determinations also is correlated with the bacteriostatic action. These em-

pirical observations must not be thought of as indicating in these cases that disinfection is due to action on the bacterial surface. With any disinfectant which acts on a mechanism located within the interior of a cell, the ability to penetrate the surface barriers is important *per se* as a factor which limits the rate at which the disinfectant can accumulate at an intracellular site of action. The most rapidly penetrating surface active bactericide could be the most potent then because it accumulates more rapidly within the organism and not because it damages the cellular membranes. As a result, each case deserves individual study before conclusions may be considered valid.

#### INHIBITION OF ENZYME ACTIVITY BY TOXIC SUBSTANCES

Interference by poisons with bacterial multiplication, growth, or metabolic activity of any kind when not caused by a primary effect on the colloidal state or structural organization of the bacteria must be due to reaction with enzymes or reactants in enzyme systems. It is with this thought in mind that the modern student of metabolism and enzymology has approached the problem of the mechanisms of disinfection. The progress which has resulted from the effort to relate specific inhibitions by toxic substances to interference with enzyme activity has made possible a sharp increase in knowledge of the mechanisms of poisoning by some few toxic agents and has led to the development of ideas and generalizations which hold increasing promise for unraveling the mysteries of disinfection.

The means by which catalytic activity may be interfered with by enzyme poisons may be classified in the following manner:

1. Destruction or alteration of an essential functional group in the protein structure of an enzyme.
2. Reduction in the effective concentration of a coenzyme or the prosthetic group of an enzyme by:
  - a. Destruction or alteration of the prosthetic group or coenzyme.
  - b. Competition between the poison and apoenzyme for the site of attachment on a coenzyme of the apoenzyme.
  - c. Competition between the poison and coenzyme for the site of attachment on the apoenzyme of the coenzyme.
3. Competition between the poison and an enzyme for the substrate of the enzyme.

Before proceeding to a more detailed consideration of these modes of enzyme inhibition it should be remarked that enzyme inhibition may be studied both *in vivo* and *in vitro*. The advantage of relative simplicity in the interpretation of data favors the *in vitro* study of the poisoning of isolated and purified enzymes. Unfortunately, the *in vitro* findings can never be considered more than suggestive of the true course of events *in vivo*.



with which latter more complex system all studies of modes of disinfection must be finally concerned.

In the never ending search for new and more efficacious disinfectants and therapeutic agents *in vitro* studies of enzymes may be useful for screening purposes but are never all-sufficient. For, while no substance active *in vivo* is inactive in some sort of *in vitro* system, it is frequently true that substances active *in vitro* are inactive *in vivo*. One cannot ignore the problems posed to the effectiveness of poisons by the selectively permeable barriers and versatile mechanisms of detoxification of organisms.

Returning now to the three ways in which enzyme systems may be inhibited by added chemicals, let us discuss first the effects of changes in the apoenzyme itself. At the outset it will be obvious that some additions to the system do not alter the apoenzyme and are without effect. Still others cause limited changes in structures having no direct role in enzymatic activity and are also without effect. As an example of the latter the addition of limited amounts of acid may be cited since the decrease in pH reflects a change in the state of ionization of some of the polar groups present. Thus the structure of the apoenzyme can be altered over somewhat restricted ranges without reducing the activity of the complete system.

However, when a chemical attacks one or more structures specifically involved either in catalytic action or as a point of attachment of coenzyme or substrate, then the extent of the inhibition can be correlated with the effect of the chemical. Indeed, theories have been presented allowing one to estimate the effect produced when the quantity of inhibitor is changed. Furthermore, it has been found that the typical inhibitions of this group may be of two types, competitive and non-competitive. The former refers to the systems in which the inhibition due to some added substance may be overcome at least in part by the addition of more of the normal substrate. In cases of non-competitive inhibition the interference cannot be reversed by adding coenzyme or substrate.

Considering the competitive type of inhibition in a little more detail, an idealized system may be developed. Without specifying actual sites of action let us take  $E$  as enzyme,  $S$  as substrate,  $P$  as products, and  $I$  as inhibitor and write the reactions involved



Enzyme and substrate combine reversibly to form a complex  $ES$  which dissociates into enzyme and products. This dissociation is written as irreversible and for practical purposes this often appears to be the case although it actually is not. In a number of situations, however, step (1) can be readily reversed to an appreciable extent by simply starting with a



mixture of enzyme and product. Yet even in these systems reaction (1) is valid as written in the early part of the process before a large amount of product has accumulated. For the present purpose we may take

$$K_{ES} = \frac{[E][S]}{[ES]} \quad (3)$$

as the equilibrium constant of the first part of (1), using the brackets to denote the molar concentration of the indicated materials.

Reaction (2) is quite analogous indicating the reversible formation of an enzyme-inhibitor complex. However, this complex is catalytically inactive and prevents the normal function of the enzyme so combined for as long as the enzyme is thus bound. The equilibrium constant is given by

$$K_{EI} = \frac{[E][I]}{[EI]} \quad (4)$$

Hence whenever  $K_{EI}$  is small, reaction (2) is displaced at equilibrium toward the inactive  $EI$ .

When  $E$ ,  $S$ , and  $I$  are present together, the relative amounts of  $ES$  and  $EI$  will depend upon four factors: the two equilibrium constants and the two concentrations. Thus, if  $EI$  is but slightly dissociated the inhibition will be relatively great when a considerable amount of inhibitor is added. However, the inhibitory effect can be reduced even then by a large addition of substrate which effectively draws  $E$  from the system (equation (3) must be obeyed) and thereby increases  $ES$ , permitting a greater formation of products. This mutual antagonism between substrate and inhibitor has led to the use of the term competitive inhibition.

The problem has been generalized quantitatively by a line of reasoning like that employed in the chapter on enzymes in the discussion of the Michaelis-Menten constant. Rather than trace the development here let us merely state the result.

$$v = \frac{V[S]}{K_{ES} + \frac{K_{ES}[I]}{K_{EI}} + [S]} \quad (5)$$

where  $v$  is the velocity of the reaction system and  $V$  is the maximum velocity attainable when inhibitor is present and when the concentration of substrate is so high that the greatest possible amount of enzyme is tied up in the enzyme-substrate complex.

If equation (8) of chapter 9 is solved for the velocity in the absence of inhibitors,

$$v = \frac{V[S]}{K_{ES} + [S]} \quad (6)$$

revealing that the presence of a competitive inhibitor in the system alters the expression for the reaction velocity by adding the term  $\frac{K_{ES}[I]}{K_{EI}}$  to the denominator. Thus we may see that if  $EI$  is relatively more stable than  $ES$ ,  $K_{EI}$  is small and  $\frac{K_{ES}[I]}{K_{EI}}$  significantly reduces the velocity. It is also evident that an increase in  $[S]$  tends to offset the effect of the inhibitor and competition results.

Many instances of competitive inhibition are known, and the phenomenon seems to be a widespread one. As an actual example, the effect of malonate on the oxidation of succinate may be cited. This case, already discussed in connection with metabolism, is typical inasmuch as malonate hinders the enzymatic oxidation of succinate. When the concentration of succinate is increased the inhibition is partly overcome.

Non-competitive inhibition is subject to the same type of analysis, but only qualitative considerations need be presented. Two things can happen. When  $K_{EI}$  of equation (4) is exceedingly small, reaction (2) proceeds very far in the direction of  $EI$  and no appreciable amount of  $I$  remains free. Therefore, the extent of inhibition is directly proportional to the amount of inhibitor added which is equal to the amount of  $EI$  formed under the conditions specified. Furthermore, the addition of substrate has no appreciable effect in such non-competitive cases because  $K_{ES}$  is very much larger than  $K_{EI}$  and the latter is very small. These two conditions mean that added substrate does not effect a reaction system whose rate depends for practical purposes only on added  $I$  and not on  $E$ .

On the other hand, non-competitive inhibition may occur when the inhibitor combines with the enzyme-substrate complex of reaction (1).



This step then effectively reduces the concentration of  $ES$  and hence inhibits the formation of products from the enzyme-substrate complex. The extent of inhibition will depend upon the dissociation constant of reaction (7), on  $K_{ES}$ , and on the quantities of  $E$ ,  $S$ , and  $I$ . Such a system is quite definitely non-competitive since the inhibitor is not competing with substrate for enzyme but rather is reacting in a subsequent step.

When mercuric ion is added to an enzyme whose activity depends upon thiol groups ( $-\text{SH}$ ), the system is inhibited. The metallic ion combines firmly with sulfur, and enzymatic action is blocked to an extent directly related to the amount of mercury added. An increase in the substrate does not affect the velocity which can be increased only by actually removing the mercuric ion or by tying it up in a compound even more stable than

that with the enzyme. This case then is a typically non-competitive inhibition.

Up to this point the discussion has been limited to inhibitions produced directly on the apoenzyme alone except for reaction (7) above. If the holoenzyme is composed of an apoenzyme and a tightly bound prosthetic group on which the inhibitor acts, the same considerations will apply and nothing more need be said. When the inhibitor acts on a coenzyme the situation is similar but complicated by the presence of an additional step. If coenzyme is represented by  $C$



The various possible complexes are indicated in the usual way by means of the appropriate letters. Reaction (8) is the normal process and may be inhibited at various steps as shown



It will be evident that additional substrate can have no effect on (9), and therefore the inhibition will be non-competitive. Reaction (10), however, will correspond to the competitive system already considered. Finally, (11) also will be non-competitive and will be analogous to reaction (7) in all respects.

In conclusion the other possible point of inhibition involves the substrate



In this instance the inhibition seemingly always must be competitive by definition because the addition of extra substrate will automatically replace that lost by reaction with the inhibitor.

#### ANTIMETABOLITES

In a strict sense the class of substances known as antimetabolites includes all compounds interfering with the utilization of metabolites. Since it has been discovered that compounds which resemble metabolites in their chemical structure may often be employed to specifically inhibit the utilization of particular metabolites the term antimetabolites has been increasingly restricted to these latter compounds. That is, the term antimetabolite has been employed to designate the inhibitory structural analogs of metabolites. This concept has had practical significance in providing for a rational approach to the synthesis of new chemotherapeutic agents.



In spite of the confusion of definition a general understanding of the main principles of inhibition induced by antimetabolites is readily outlined. In the first place the mechanisms of inhibition will be found among those discussed in the preceding section in connection with enzymes. At present there is no reason to assume that inhibition by antimetabolites is in any way peculiar except possibly that this inhibition is limited to the specific reaction systems in which the given metabolites participate.

Two types of direct antagonism are recognizable; inhibition of either the synthesis or the direct utilization of a metabolite. Illustrating in generalized form



where  $A$ ,  $B$ , and  $C$  are reactants,  $M$  is the essential metabolite, and  $P$  represents the products. Whenever the antagonist acts on reaction (1) to prevent the formation of  $M$ , the activity of the system (an organism, a tissue, an isolated system of enzymes) is affected to the extent that  $M$  is required by that system.

The exceedingly complex and versatile living organism may substitute for this metabolite or may be able to operate by way of some other metabolic pathway. If it can do neither it ceases to grow and perhaps dies. However, if an external source of  $M$  is provided, normal operations are resumed granting that the metabolite can penetrate to the zone where it is consumed ordinarily. In such cases there can be no competition between the antimetabolite and added metabolite, for the antagonism precedes  $M$  and effects the synthesis only. Hence when  $M$  is added the external source is directly utilized by the ordinary pathway, and  $M$  only needs be supplied in the normal amounts in order to produce a typical response.

On the other hand reaction (2) serves to illustrate the effect on the utilization of a metabolite. In this case the addition of  $M$  will reverse the antagonism if the system is competitive, and it will do so to the extent predicted according to the methods of the preceding section. Whenever the antagonism is non-competitive, the degree of inhibition of the metabolism is determined only by the amount of antimetabolite.  $M$  does not reverse the inhibition, nor does the enzyme system operate. This type of effect is characteristic of the substances like mercuric ion which are not usually regarded as antimetabolites since the antagonism shown cannot be reversed by the addition of metabolite.

Actually it is quite obvious that a given antagonist can inhibit the utilization of one metabolite and the synthesis of another following in a reaction chain so that both actions are manifested in one system. For

example,  $\beta$ -aminobutyric acid inhibits the growth of yeast by interfering with the synthesis of pantothenic acid from  $\beta$ -alanine. If  $\beta$ -alanine is added a competitive reversal of the antagonism is observed. When enough pantothenic acid is added to supply the normal concentration, the presence of large amounts of  $\beta$ -aminobutyric acid has no effect, and the reversal is non-competitive.

A great variety of antimetabolites are now known that interfere in systems involving amino acids, histamine, various vitamins, metabolic intermediates like succinate, and so on. Some are of practical value as chemotherapeutic agents, and others have been valuable aids in unraveling the reaction patterns of biological systems. It might be well to point out in conclusion that normal organisms utilize metabolites which mutually antagonize each other. Thus a system of checks and balances can be maintained. Such mutual interference is observed with amino acids for example, and may be illustrated with *Lactobacillus casei* whose utilization of glutamine is antagonized by aspartic acid. Asparagine and glutamate as well as glutamine overcome the effect of the aspartate indicating that this organism like others is sometimes able to substitute metabolites when an antagonist is present.

## EXAMPLES OF DISINFECTION BY SPECIFIC AGENTS

### TOXICITY OF HYDROGEN ION

The growth of each species of bacteria is characterized by a limited range of hydrogen ion ( $\text{H}_3\text{O}^+$ ) concentration within which it can take place. Additionally, at sufficiently high concentrations of  $\text{H}_3\text{O}^+$  the viability of bacterial cultures is reduced and sterilization accomplished. Since the surface composition of bacteria includes amphoteric substances it is possible that the state of dissociation of these constituents of the surfaces responsive to the presence of  $\text{H}_3\text{O}^+$  affects properties of permeability. Thus indirectly hydrogen ions could act upon metabolic and growth processes by affecting the permeability of bacteria. In this connection the state of dissociation of nutrient materials as influenced by the  $\text{H}_3\text{O}^+$  might also be independently important in determining the transfer of nutrients across bacterial surfaces.

It has been suggested that  $\text{H}_3\text{O}^+$  may interfere with bacterial processes by competing with ionic nutrients for cellular sites of adsorption. In Table 56, data are presented that show the preferential adsorption of  $\text{H}_3\text{O}^+$  by *Escherichia coli* in the presence of nutritionally important inorganic electrolytes. As a further test of this hypothesis it would be desirable to know how the bacteria that are tolerant of acid, for example a species such as *Thiobacillus thiooxidans* which actually prefers to grow at pH values close to 2, compare in the displacement of adsorbed cation by  $\text{H}_3\text{O}^+$  with such

species as *Escherichia coli* which demands a considerably higher pH for growth and survival.

At high enough concentrations it is quite probable that the hydrogen ion is toxic because of the direct hydrolysis of sensitive proteins and nucleic acids located at the bacterial surface. Unfortunately practically no data exist which would permit one to estimate the actual importance of this mechanism.

Comparison of strong and weak acids reveals that undissociated molecules may be toxic apart from any toxic activity of the  $\text{H}_3\text{O}^+$ . As a rule, organic acids are more toxic than strong acids at the same hydrogen ion concentration. No doubt this is in part a reflection of the fact that organic

TABLE 56

*The displacement of  $\text{H}_3\text{O}^+$  from Escherichia coli by some electrolytes*

SALT	pH		mE $\text{H}_3\text{O}^+$ DISPLACED FROM 100 GRAMS OF BACTERIA	ADSORBED $\text{H}_3\text{O}^+$ DISPLACED %
	Salt solution	Bacteria* + salt solution		
NaCl.....	5.2	4.56	0.21	0.94
KCl.....	5.43	4.56	0.21	0.94
$\text{CaCl}_2$ .....	5.00	4.17	0.71	3.10

\* Bacteria in original suspension mixed with the salt solution had 22.3 milliequivalents (mE) of adsorbed  $\text{H}_3\text{O}^+$  at pH 5.0.

The data show that the sodium, potassium, and calcium cations are not effective in displacing adsorbed  $\text{H}_3\text{O}^+$ . Conversely  $\text{H}_3\text{O}^+$  is capable of replacing these ions at adsorption sites without difficulty.

(Data from McCalla, 1941.)

acids penetrate biological membranes as undissociated molecules. An explanation of this sort still leaves unanswered the question of the mechanism of toxicity of the acid which has penetrated into the interior of the organism. It is not known whether the mechanism of poisoning involves a harmful change in the intracellular pH.

In the case of nitric and hydrofluoric acids the anion appears to be toxic so that the total toxicity of the acid is a summation of the effect exerted both by the hydrogen ion and the anion.

#### TOXICITY OF HYDROXYL ION

Alkalies have proven to be efficient and economical disinfectants for practical use. Strong alkali will hydrolyze many proteins and nucleic acids at room temperature. A sufficiently high hydroxyl ion ( $\text{OH}^-$ ) concentration is thus certain to harm organisms by direct reaction with physiologically



active cellular constituents and structures. The acid fast bacteria are exceptional in their relative resistance to strong alkalis. Advantage is taken of this fact in the isolation of cultures of acid-fast bacteria from mixed cultures or from the flora of natural environments by the use of alkali to selectively destroy the non-acid fast organisms. No proven explanation of the alkali resistance of acid-fast bacteria exists though peculiarities of surface structure and chemical composition of these organisms obviously must be involved.

The potency of alkalis as disinfectants is a function of  $\text{OH}^-$  concentration. Thus, at a fixed temperature and pH the amount of alkali does not affect the velocity of disinfection. In the case of an alkali such as barium hydroxide, possessing a toxic cation, the total activity of the alkali is a summation of the activities of the cation and hydroxyl ion.

With sodium hydroxide, disinfection over the temperature range between 2 and 25°C. is independent of temperature. McCulloch has shown this to be due to the fact that in this temperature range the ratio of  $\text{H}_3\text{O}^+$  activity (thermodynamic) to  $\text{OH}^-$  activity decreases with an increase in temperature with the result that the greater activity of the  $\text{OH}^-$  over the  $\text{H}_3\text{O}^+$  activity at lower temperatures balances the decrease in germicidal efficiency of each  $\text{OH}^-$  expected at the lower temperature. This fact has practical significance since it makes possible the useful application of cold sodium hydroxide under field conditions where heating the disinfectant would be inconvenient.

In the case of bacterial spores there is evidence which has been interpreted as indicating that the alkali penetrates the organism as the undissociated molecule. This is based on experiments depending on the addition of a common ion to suppress the ionization of the alkali. The addition of the common ion seems to increase the ability of a fixed concentration of alkali to kill the spores. While one may accept the results of the addition of a common ion, one may wonder about the interpretation. It is difficult to conceive how the addition of a common ion such as  $\text{Na}^+$  can suppress to any significant extent the ionization of so strong an alkali as sodium hydroxide.

The spore coats probably are not metabolically active structures. As a result damage to the spore coat by  $\text{OH}^-$  may be less important to the spore than equivalent damage to the metabolically active cell wall of vegetative forms of bacteria.

#### TOXICITY OF MERCURIC ION

Ionizable mercury compounds poison biological systems by release of the mercury ion which reacts with cellular constituents. No significant knowledge exists of the mechanisms of action of organic mercurial disinfectants in which the mercury cannot ionize. When the commonly used disinfectant mercuric chloride is added to a bacterial suspension both growth and re-

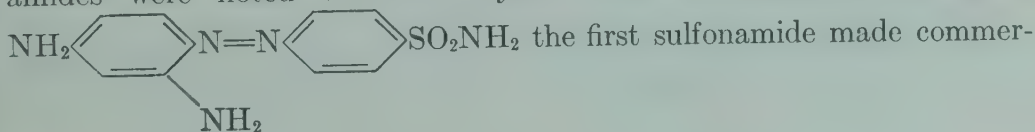
production are adversely affected, and either a bacteriostatic or bactericidal effect ensues depending on such factors as the length of time of exposure, the concentration of disinfectant, and the temperature.

The basic reaction of poisoning is the combination of the mercuric ion with sulfhydryl groups. Inasmuch as the catalytic activity of numerous enzymes is dependent on the presence of free sulfhydryl groups, the mercuric ion acts as a disinfectant by interfering with enzyme activity. It is not known whether the poisoning by the mercuric ion involves a primary site of action on a particularly sensitive and essential enzyme or whether it is the summation of the effects of reaction with numerous enzymes depending upon sulfhydryl groups.

Bacteria exposed to mercuric chloride for only a short time will regain their respiratory activity and capacity for growth if they are merely washed repeatedly with water or an isotonic salt solution. Beyond the time when such washing is sufficient to reverse poisoning by mercuric chloride, the addition of compounds such as hydrogen sulfide and organic substances with free sulfhydryl are capable of reversing the poisoning. The reversal of sulfhydryl is specific and can be followed quantitatively. The reaction between glutathione and mercuric ion is stoichiometric, that is, in the expected molar ratio of two sulfhydryl groups to one mercuric ion. These observations suggest that the disinfecting action of the mercuric ion is separable into three phases, a stage of diffusion into the bacilli which is reversible by simple dilution, a second stage of reaction with sulfhydryl groups which is reversible in the presence of substances capable of competitively displacing the mercuric ion, and a final irreversible stage. Exactly what happens in the latter stage is uncertain. The probability is that the reaction of the mercuric ion with an increasing number of sulfhydryl groups finally results in an irreversible change in the state of essential proteins, i.e., precipitation to solid phase, with which the soluble specific sulfhydryl containing antidotes are not capable of reaction. Such changes in state would affect the colloidal structure of protoplasm and might well be harmful apart from the inactivation of particular sulfhydryl containing enzymes. It should also be remarked that the mercuric ion is capable of reaction with ionized carboxyl groups, and this too must contribute to the total picture of toxicity.

#### THE ANTIBACTERIAL ACTIVITY OF SULFONAMIDES

Historically, the initial observations of antibacterial activity by sulfonamides were noted with azo dyes of these compounds. Prontosil,





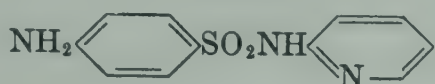
cially available for therapeutic uses, soon after its introduction was shown to be reduced *in vivo* and the antibacterial activity to be associated solely with sulfanilamide,  $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NH}_2$ , one of the products of the reduction. The term sulfonamide designates a class of compounds,  $\text{R-SO}_2\text{NH}_2$ , which includes inhibitors of bacterial activity and inactive substances as well. The aryl sulfonamides have proven to be those of most practical interest in the treatment of infectious diseases. The term aryl is used to designate aromatic substituents: phenyl, *p*-aminophenyl, naphthyl, and so on. Sulfanilamide, which may be considered the prototype of the aryl sulfonamides, is rendered innocuous for bacteria if the amino group is shifted from the para to the meta or ortho positions. The amino group appears necessary for antibacterial activity since with few exceptions its replacement by methyl or other organic substituents leads to a loss of activity. When the amino group is itself modified antibacterial activity may be unaffected, enhanced, or completely lost. If the compounds inactive *in vitro* can be reduced or otherwise attacked to yield a free amino or nitro group, antibacterial activity is restored. The acetyl and alkylated derivatives of active sulfonamides, for example, have been shown to be bacteriostatic *in vivo* by reason of their deacetylation and dealkylation respectively by animal tissue to the free sulfonamide.

When the amino group of sulfanilamide is oxidized as in  $\text{NHOH-C}_6\text{H}_4\text{SO}_2\text{NH}_2$  or separated from the benzene ring by carbon atoms as in marfanil,  $\text{NH}_2\text{CH}_2\text{C}_6\text{H}_4\text{SO}_2\text{NH}_2$ , antibacterial activity may be retained. However, the bacteriostatic properties of these compounds unlike sulfanilamide are not antagonized by *p*-aminobenzoic acid. For this reason, it has not been customary to refer to these compounds as *true sulfonamides*. A true sulfonamide has been defined as one which resembles sulfanilamide in its biochemical activity, the criterion of this resemblance being the ability of *p*-aminobenzoic acid to antagonize the bacteriostatic property of the drug. In common parlance the use of the word sulfonamide implies a reference to a true sulfonamide. Accepting this definition the  $\text{NH}_2\text{C}_6\text{H}_4\text{S}$  portion of the molecule appears basic for sulfonamide activity. The sulfur atom cannot be replaced by carbon as the resulting carboxylic amides while they may be active against bacteria are not antagonized by *p*-aminobenzoic acid, as is true also for the replacement of the sulfur by selenium or tellurium.

Substitution of a hydrogen atom on the nitrogen attached to the  $\text{SO}_2$  group has resulted in the synthesis of a number of clinically useful powerful bacteriostatic agents differing in such physical properties as solubility, and



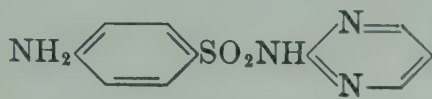
in pharmacological properties such as the ability to be absorbed from the alimentary tract and toxicity for animal tissue. Among the more widely employed of these true sulfonamides are the following N'-substituted derivatives of sulfanilamide:



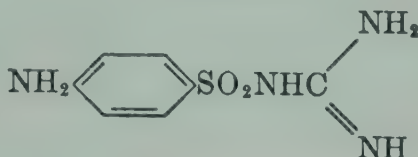
Sulfapyridine



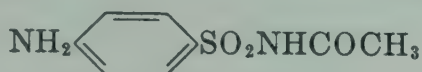
Sulfathiazole



Sulfadiazine



Sulfaguanidine



Sulfacetimide

The sulfamyl groups of sulfanilamide and the N'-monosubstituted derivative are capable of ionization:  $\text{—SO}_2\overset{\text{H}}{\underset{|}{\text{N}}}\text{R} \rightleftharpoons \text{—SO}_2\overset{-}{\text{N}}\text{R} + \text{H}^+$ . In seeking for a theoretical basis for guidance in the synthesis of new therapeutically effective drugs evidence has been obtained suggesting that the more negative the sulfamyl group the greater the bacteriostatic potency. The increased bacteriostatic potency has been said to be a reflection of the fact that the more negative the sulfamyl group the more nearly the compound resembles *p*-aminobenzoic acid which at physiological values of pH is almost completely ionized. Presumably, the *in vivo* competition of sulfonamides with *p*-aminobenzoic acid would be between their ionic forms.

At a fixed pH value the molar concentrations of various sulfa drugs required to induce the same level of bacteriostasis is dissimilar. But when the differences in extent of dissociation are taken into account the drugs are shown to possess about the same activity. The minimum quantity of *p*-aminobenzoic acid completely preventing bacteriostasis by a given quantity of ionized sulfonamide is about the same for the different sulfonamides (Table 57). These facts rule in favor of the views that the ionized state of sulfonamides is bacteriostatically active and that there is a common mechanism of bacteriostasis by the various true sulfonamides.

Actually when the bacteriostatic potency of a sulfonamide at different pH values is determined, it is generally found that the maximum activity is exhibited near the  $\text{pK}_a$  value of the drug. With a series of different sulfonamides tested at a given pH, the drug with a  $\text{pK}_a$  value closest to the

TABLE 57

*Antagonism of p-aminobenzoic acid for different sulfonamides*

DRUG	RATIO OF p-ABA TO TOTAL DRUG AT pH 7	RATIO OF p-ABA TO DISSOCIATED DRUG AT pH 7
Sulfanilamide.....	1:5000	1:1.4
Sulfapyridine.....	1:40	1:1.4
Sulfathiazole.....	1:8	1:4.9
Sulfadiazine	1:8	1:6.4

The ratios represent the minimum molar concentration of *p*-aminobenzoic acid required to prevent bacteriostasis by the sulfonamide. The tests were conducted at pH 7, at which value *p*-aminobenzoic acid would be largely dissociated and the sulfonamides would each be dissociated to a different extent.

(Data from Fox and Rose, 1942.)

TABLE 58

*Relation of the bacteriostatic potency of sulfonamides to pK<sub>a</sub> values employing Escherichia coli as the test organism grown in a p-aminobenzoic acid-free synthetic medium*

SULFA	pK <sub>a</sub>	CONCENTRATION REDUCING GROWTH RATE 85%
Sulfanilamide.....	10.4	50 × 10 <sup>-4</sup> M
Sulfapyridine.....	8.4	25 × 10 <sup>-4</sup>
Sulfathiazole.....	7.1	5 × 10 <sup>-4</sup>
Sulfadiazine.....	6.5	10 × 10 <sup>-4</sup>

The initial pH of the medium was 7.2 before autoclaving and during the course of growth the pH dropped to acidic values of pH 6 to 5.3.

(Data of bacteriostasis from Kohn and Harris, 1941.)

TABLE 59

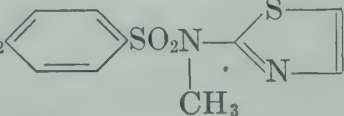
*The relation of the pK<sub>a</sub> value of sulfonamides to the antibacterial index in the presence of p-aminobenzoic acid*

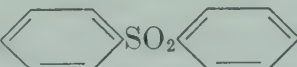
DRUG	pK <sub>a</sub>	RECIPROCAL OF THE ANTIBACTERIAL INDEX			
		6	7	8	9
Sulfanilamide.....	10.4	13,440	2,500	291	50.6
Sulfapyridine.....	8.4	650	123	45.8	22.9
Sulfathiazole.....	7.1	71	22	34.2	45.7
Sulfadiazine.....	6.5	34	67.2	114	171
Sulfacetamide.....	5.4	169	158	244	355
Methyl sulfathiazole	Non-ionic	6000	1000	200	44

The antibacterial index has been defined by Mellwain (1949, *Science* 95: 509-511) as the ratio of the molar concentration of an antagonist to the molar concentration of a drug causing a minimal bacteriostasis.

(Data taken from Northey, 1948, tables 319, 320.)

pH of the test exhibits the greatest bacteriostatic activity (Table 58). In Table 59 data are presented which show that the amount of *p*-aminobenzoic acid required to antagonize a particular sulfonamide is greatest at a pH value near the  $pK_a$  value of the sulfonamide. Similarly at a given pH value the greatest amount of *p*-aminobenzoic acid required for complete antagonism is for the sulfonamide whose  $pK_a$  value is closest to the pH of the test solution. It should be recalled that the  $pK_a$  value of an acid is equal to the logarithm of the reciprocal of the dissociation constant or is the pH at which exactly half of the molecules in solution exist in the ionized form. Consequently, it appears that both the ionized and non-ionized molecular states contribute to the bacteriostatic potency of sulfonamides. To account for this fact in the face of evidence favoring the view of competition taking place between the ionic states of sulfonamides and *p*-aminobenzoic acid, it has been postulated that bacteria are permeable only to the unionized sulfanilamide molecule. After passage through the limiting membranes, ionization of the sulfonamide occurs, and in this form the drug competes for specific enzyme receptor sites involved in the normal metabolic utilization of *p*-aminobenzoic acid.

Since non-ionizing true sulfonamides (in the chemotherapeutic sense) are known such as *N*'-methyl sulfathiazole,  $\text{NH}_2$ - and

diaminodiphenylsulfone,  $\text{NH}_2$ -- $\text{NH}_2$ , which are as potent as some ionizing sulfonamides and more potent than others, it is doubtful that the permeability factor is the only role in bacteriostasis of the non-ionic state of sulfonamide. At the present stage of knowledge, it is logical to accept both the ionized and undissociated states as being bacteriostatically active.

Non-ionizing sulfonamides are more bacteriostatic at alkaline than acid pH values (Table 59). Wyss has suggested that this may be due to a metabolic need for larger quantities of *p*-aminobenzoic acid at higher pH values since those bacteria requiring *p*-aminobenzoic acid as a growth factor grow less with fixed and limiting amounts of *p*-aminobenzoic acid in media at alkaline than at acidic values of pH.

### *Bacteriostasis by Sulfonamide*

The sulfonamides have proven to be bacteriostatic to all species which have been tested. Typical growth curves of cultures grown in the presence of a sulfonamide are illustrated in Fig. 100. Three effects of the sulfonamide may be noted:

- 1) Inhibition of the multiplication rate is directly related to the concentration of the sulfonamide.



2) A decreased total population results, and the decrease, too, is related directly to the concentration of the sulfonamide (Table 60).

3) There is a delay in the action of the sulfonamide. This delay is related to the cultural age of the inoculum since it is apparent only when the inoculum is taken from an actively multiplying culture.

The great mass of observations with the sulfonamides employed therapeutically have dealt with bacteriostatic effects. Nonetheless, under ap-

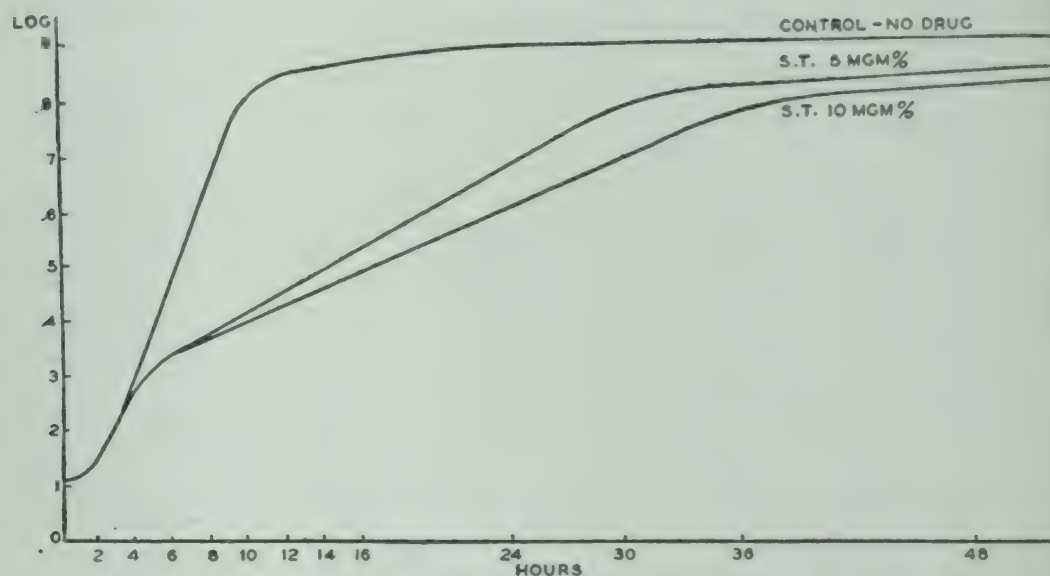


FIG. 100. Growth curves of cultures of *Salmonella enteritidis* with varying concentrations of sulfathiazole.

(From Muir, Shamleffer, & Jones, 1942)

The expression *milligrams percent* means the amount of drug in milligrams per 100 ml. and is a method of expressing concentrations more common among pharmacologists than among bacteriologists and chemists. This term is in common use in pharmacology even though of somewhat ambiguous origin. It would appear to be more logical to use millipercents when expressing concentrations in this manner.

proprate conditions sulfonamides can be shown to be bactericidal. At temperatures at which most bacteriological studies are conducted, the sulfonamides are not very soluble in either water or bacteriological media. Thus, the range of concentrations that can be studied may not often extend to high enough values for bactericidal effects to be noted.

The bacteriostatic activity of sulfonamides has a positive temperature coefficient. A sufficient rise in temperature can result in a bactericidal action by sulfonamides which is not merely due to an additive effect of any harm induced by the rise in temperature itself.

The reduction noted in total population in a medium with a fixed concentration of sulfonamide inoculated with a decreasing number of inoculum

organisms (Table 61) is an unusual observation. Since the phenomenon occurs with inocula washed free of media, the cause of this observation cannot be explained by the existence of variations in the amount of extra-cellular antagonists transferred with varying quantities of inocula. Two hypotheses appear attractive, one, that the level of inhibition is directly

TABLE 60

*Relation of the number of colonies of Streptococcus pyogenes developing in blood-agar medium to the concentration of sulfanilamide*

MOLAR CONCENTRATION OF DRUG ( $\times 10^4$ )	NO. OF COLONIES	INHIBITION
		%
0	206	0
0.6	107	48
6.0	92	55
60	91	56
75	84	60
100	79	62
150	59	71
300	52	75

(From Mellon, Gross, and Cooper, 1938.)

TABLE 61

*Effect of variation in the number of organisms in the inoculum on the per cent of the total population attained in a medium containing a sulfonamide*

ORGANISM	DRUG	CONTROL POPULATION WITH AN INOCULUM OF		
		$50 \times 10^6$	$25 \times 10^6$	$12.5 \times 10^6$
		%	%	%
Pneumococcus, Type II..	Sulfapyridine	61	51	44
Paratyphoid A.....	Sulfapyridine	77	57	46
Streptococcus pyogenes....	Sulfanilamide	68	57	34

(From Libby, 1940.)

related to the number of molecules of sulfonamide per bacterium, and second, that bacteria exposed to sulfonamides can utilize a store of intracellular metabolites which permits them to multiply a definite number of times before the bacteriostatic action of the drug can express itself.

#### *Mechanism of Bacteriostasis by Sulfonamide*

All the true sulfonamides probably cause bacteriostasis by the same mechanism, one which depends on a disturbance in the normal metabolic utilization of *p*-aminobenzoic acid. This similarity is shown by the fact that

though bacteria vary in their sensitivity, the relative order of toxicity of the sulfonamides for various bacterial species is the same. *p*-Aminobenzoic acid can completely suppress bacteriostasis at all concentrations of a given sulfonamide, the molar ratio of *p*-aminobenzoic acid to sulfonamide required for the antagonism being the same at all concentrations of the sulfonamide. In consonance with the notion that all true sulfonamides act on the same cellular loci as competitors of *p*-aminobenzoic acid, the ratios of the amounts of different sulfonamides required to overcome the antagonism of a fixed concentration of *p*-aminobenzoic acid is the same with different organisms.

TABLE 62

*Neutralization of the antagonism of p-aminobenzoic acid by sulfanilamide and sulfathiazole with different bacteria*

ORGANISM	MOLAR RATIOS		
	Sulfanilamide/ <i>p</i> -ABA	Sulfathiazole/ <i>p</i> -ABA	(Sulfanilamide/ <i>p</i> -ABA)/ (Sulfathiazole/ <i>p</i> -ABA)
<i>Escherichia coli</i> .....	2000	27	74
<i>Aerobacter aerogenes</i> .....	3220	45	72
<i>Staphylococcus aureus</i> .....	4660	53	88
<i>Pseudomonas aeruginosa</i> .....	13,330	184	73
<i>Salmonella typhimurium</i> .....	6650	92	72
<i>Lactobacillus acidophilus</i> .....	8000	133	60
<i>Proteus vulgaris</i> .....	4000	55	73

(From Wyss, Grubaugh, and Schmelkes, 1942.)

Data comparing sulfanilamide and sulfathiazole in this respect are presented in Table 62.

Another potent argument in favor of the similarity in the bacteriostatic mechanism of the different true sulfonamides is that with few known exceptions the development of resistance by bacteria to one of the drugs results in the simultaneous acquisition of resistance to all others but not to those sulfonamides which are not antagonized by *p*-aminobenzoic acid.

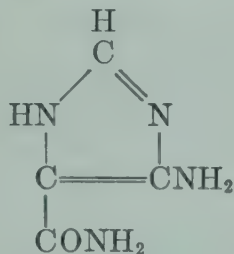
Sulfonamides inhibit not the synthesis but rather the utilization of *p*-aminobenzoic acid. It has already been mentioned that the amount of *p*-aminobenzoic acid which must be added in order to get growth and multiplication in a bacterial culture containing sulfonamide is in a fixed ratio to the amount of a particular sulfonamide present. This competitive relationship would not be expected if the sulfonamide interfered with the synthesis of *p*-aminobenzoic acid. Under a given set of circumstances the threshold or minimum concentration of a growth factor required for growth



and multiplication is a fixed quantity and is independent of the amount of any inhibitor which merely prevents the synthesis of the growth factor. The very fact that sulfonamides can prevent the growth of bacteria unable to synthesize *p*-aminobenzoic acid and which must have an external source of this metabolite argues that the primary action of sulfonamides is directed against the utilization of *p*-aminobenzoic acid.

The competition of true sulfonamides with *p*-aminobenzoic acid is for unoccupied cellular receptor sites. It does not involve a displacement of any *p*-aminobenzoic acid which is already bound to an apoenzyme or which is incorporated as a portion of more complex metabolites prior to the addition of a sulfonamide. This absence of rapid displacement probably accounts for the delay often observed in bacteriostasis by sulfonamide and also for the lack of any leakage or displacement of *p*-aminobenzoic acid from bacilli suspended in sulfonamide solutions.

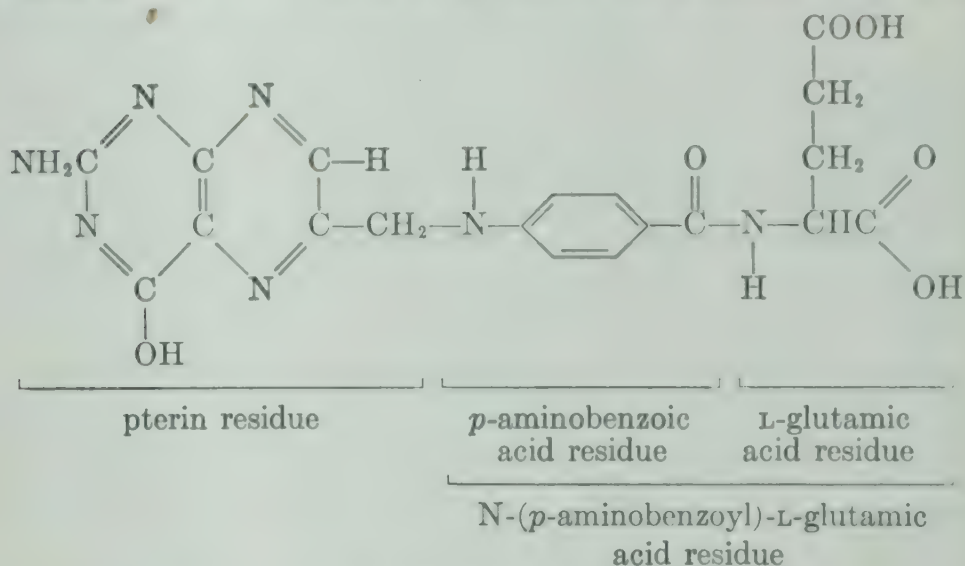
During bacteriostasis produced by sulfonamide a possible precursor of purines having the following structure accumulates:



It has also been observed that *Acetobacter suboxydans*, which must be supplied with *p*-aminobenzoic acid as a growth factor, requires much less *p*-aminobenzoic acid for growth in synthetic media to which purines have been added. Purines are also known to non-competitively inhibit sulfonamide bacteriostasis. These observations have been explained by assuming that one of the metabolic functions of *p*-aminobenzoic acid is as a coenzyme in systems synthesizing purines.

Still another antagonist of sulfonamide bacteriostasis is the amino acid methionine. Unlike the antagonism shown by *p*-aminobenzoic acid, methionine is effective only over a limited range of concentrations ( $10^{-5}$  to  $3 \times 10^{-4}$  M). The ratio of this amino acid to sulfonamide for complete abolition of bacteriostasis with different concentrations of sulfonamide is not constant, so that a competitive relationship does not exist. Mutant strains of *Escherichia coli* having a nutritional requirement for *p*-aminobenzoic acid are able to grow in a mixture of purines, thymine, and methionine as a replacement for the *p*-aminobenzoic acid. These findings indicate an involvement of *p*-aminobenzoic acid in the synthesis of metabolites acting as non-competitive types of antagonists for sulfonamides.

*p*-Aminobenzoic acid occurs as a part of the structure of folic acid:



Sulfonamides will prevent the synthesis of folic acid by organisms which do not require this B vitamin as a nutrient. N-(*p*-aminobenzoyl)-L-glutamic acid is eight to ten times as effective as *p*-aminobenzoic acid as an antagonist of the bacteriostasis of sulfonamides. On the other hand, organisms which do not synthesize folic acid and which are supplied it for growth, tend to be highly resistant to bacteriostasis by sulfonamides. Therefore, it is logical to believe that sulfonamides prevent the incorporation of *p*-aminobenzoic acid into folic acid and thus interfere with metabolic reactions which involve folic acid as coenzyme.

Folic acid cannot replace *p*-aminobenzoic acid as a growth factor for organisms requiring *p*-aminobenzoic acid. Nor can it take the place of *p*-aminobenzoic acid required by *Escherichia coli* for the synthesis of methionine and purines. Consequently, it appears that *p*-aminobenzoic acid plays a number of roles in metabolism not all of which are the result of its occurrence as a portion of the structure of folic acid. Interference with the metabolism of folic acid by competition for loci on enzymes involved in the insertion of *p*-aminobenzoic acid into the structure of folic acid cannot be an all-sufficient explanation of the mode of action of the true sulfonamides.

Sulfonamides interfere with bacterial respiration, but the concentrations required are greater than those which need to be employed for inducing bacteriostasis. The lack of parallelism between the inhibition of respiration and of multiplication is confirmed by other kinds of evidence. For example, the respiration of a strain resistant to sulfonamide and the respiration of a strain of *Escherichia coli* sensitive to sulfonamide were found to be equally susceptible to sulfanilamide. This qualitative difference existed even though

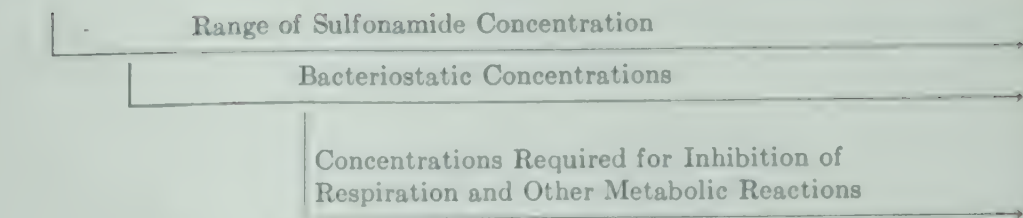
the concentration of sulfanilamide actually required to prevent the multiplication of the resistant strain was twenty times that required for the sensitive strain.

Sulfonamides are adsorbed by various organic colloidal materials, plasma proteins, activated carbon, and various inorganic catalysts. In these cases the uptake of the sulfonamide may often be shown to be inhibited by the presence of *p*-aminobenzoic acid. There are, however, also situations where true sulfonamides and *p*-aminobenzoic acid do not compete for cellular substrates. As one example, phenol oxidase will act on *p*-aminobenzoic acid without interference by sulfonamides. Alternatively, *p*-aminobenzoic acid does not prevent sulfanilamide from interfering with the combination of coenzyme II with the yeast apoenzyme active in the oxidation of glucose-6-phosphate to 6-phosphogluconate.

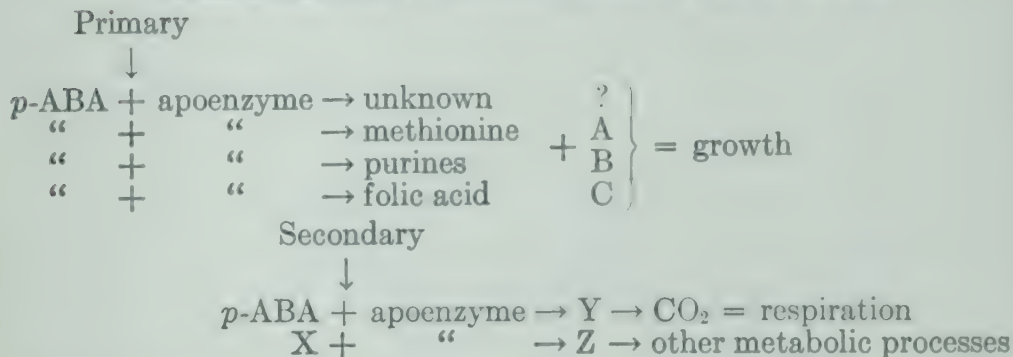
From the data cited it is evident that the chemical and structural organization of the bacterial organism presents numerous possibilities for the binding or reaction of sulfonamides with metabolically active cellular substrates. But in an explanation of the bacteriostasis caused by sulfonamides, it is the mechanism of activity of the lowest inhibitory concentrations of sulfonamides for growth which must be considered as of primary importance. Inasmuch as different cellular substrates or loci will have varying degrees of affinity for a sulfonamide it is those most avid systems which are of primary importance. These apparently involve enzymatic sites at which *p*-aminobenzoic acid will be combined probably as coenzyme with apoenzymes to form holoenzymes which function in anabolic reactions. As the concentration of a sulfonamide is increased, an increasing number of different kinds of potentially reactive loci will take up sulfonamide and an increasing number of measurable cellular functions will be disturbed as threshold values for inhibition of the individual functions are exceeded. This concept may be summarized in a diagram modified from Kohn (1943). The diagram is also intended to emphasize that many details of the biological activity of sulfonamides remain to be elucidated, see page 644. It can be seen that at lower concentrations of sulfonamide, reactions involving growth are inhibited, while specific reactions involving respiration and other catabolic mechanisms are inhibited only at higher concentrations.

Two intriguing unexplained anomalies in the relationship of *p*-aminobenzoic acid to sulfonamide bacteriostasis deserve mention as examples of apparent exceptions to the competitive metabolite theory of sulfonamide activity. Tamuri (1944) has found that sulfapyrazine, sulfathiazole, and sulfadiazine will inhibit the multiplication of *Pasteurella tularensis* *in vitro* without interference by *p*-aminobenzoic acid. Tobie and Jones (1949) report that 2-hydroxy-4-aminobenzoic acid prevents the utilization of *p*-aminobenzoic acid by the tubercle bacillus but not by other bacteria. Yet, mu-





Sites of Action of Sulfonamide for Harmful Effects:



tants of *Escherichia coli* requiring *p*-aminobenzoic acid will use this compound as a growth factor in place of *p*-aminobenzoic acid.

### Resistance to Sulfonamides

Two biochemical mechanisms of resistance to sulfonamides have been supported by experimental data, namely, an increased capacity for the synthesis of *p*-aminobenzoic acid and a more efficient utilization of *p*-aminobenzoic acid. The latter was deduced from the observation that a parent bacterial strain, in which one mole of *p*-aminobenzoic acid antagonized 14 moles of sulfathiazole, gave rise to a resistant strain in which one mole of *p*-aminobenzoic acid successfully antagonized 220 moles of sulfathiazole. Little consideration has been given to the possibility that a change in permeability properties might account for increased resistance to the extent that bacteriostasis is concerned with intracellular processes. A change in permeability properties might be of particular importance in those exceptional cases where resistance to one sulfonamide does not result in an equivalent increase in resistance to other sulfonamides.

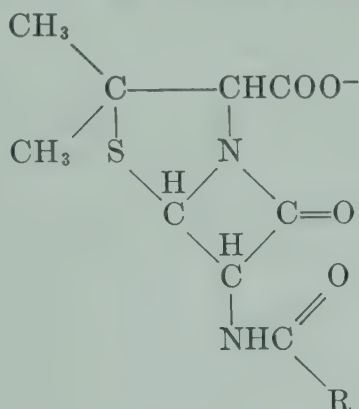
Bacteriologists have devoted little study to the possible existence of specific biochemical mechanisms of detoxification for disposal of sulfonamides by resistant bacteria.

### PENICILLIN

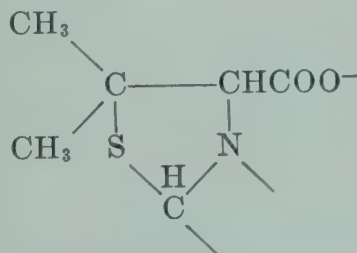
*Antibiotics*, substances of biological origin which interfere with the growth or other metabolic activities of organisms, have been known for many years. But it was the discovery of penicillin (1928) and its eventually successful

clinical use and large scale commercial production (1941–1945) that provided the impetus to the modern intensive study of antibiotics. The historical search for effective chemotherapeutic agents has resulted in an overwhelming concern with antibiotics in the treatment of infectious diseases. In common parlance, therefore, the term antibiotic has become restricted to substances interfering with the biological activities of pathogenic microorganisms.

Penicillin is an antibiotic produced by a variety of fungi as well as by members of the *Penicillium notatum-chrysogenum* group from which it was originally described. Actually the term penicillin is used to designate a group of related substances of the following chemical composition:



The four atom ring, the  $\beta$ -lactam group, was found in natural compounds for the first time in penicillin. The antibacterial activity of penicillin is destroyed when the  $\beta$ -lactam group is ruptured, and the ease with which this ring structure is broken is responsible for the instability of penicillin noted in the early attempts at isolation and purification of penicillin from cultures. The chemotherapeutic activity is also dependent on the maintenance of the thiazolidine ring,



and on the optical configuration of the penicillamine (D- $\beta$ -thiovaline) component of the penicillin molecule. The nature of the side chain (R) on the penicillin molecule does not affect seriously the antibacterial activity but is important for the pharmacological properties such as rate of secretion

into urine. In Table 63 the historically important penicillins and some of their properties are listed. The nature of the side chain occurring in penicillin can be influenced by adding particular organic constituents to media employed for the growth of the fungi producing penicillin. Apparently many organic substituents of culture media can be directly incorporated into the penicillin molecule. This fact has been used on a practical scale to influence the nature of the penicillin synthesized by the fungi during commercial production and thus to increase the relative yields of the desired end product. The production of benzyl penicillin (penicillin G), which of

TABLE 63  
*Properties of some common penicillins*

R SUBSTITUENT		MOL WEIGHT OF THE Na SALT	pK <sub>a</sub>	In Vitro ACTIVITY	
Chemical name	Symbolic designation			<i>Staphylococ- cus aureus</i>	<i>Bacillus subtilis</i>
				units/mg*	
Benzyl.....	G	356.2	2.5	1,667	1,667
p-Hydroxybenzyl.....	X	372.2	2.5	900	1,500
Δ <sup>2</sup> -Pentenyl.....	F	334.2	2.7	1,500	970
n-Amyl.....	Dihydro F	335.2	2.7	1,670	660
n-Heptyl.....	K	364.3	5.0	2,300	700

\* A unit of penicillin is the amount of substance just sufficient to inhibit the growth of *Staphylococcus aureus* in 50 ml. of nutrient broth under specified conditions.

1 unit of benzyl penicillin = 0.6 μg

1 gram of benzyl penicillin = 1,666,666 units

1 mole of benzyl penicillin =  $583.3 \times 10^6$  units

all the penicillins has found the greatest use in clinical medicine, is directed and increased by the addition of phenylacetamide to the culture medium.

Penicillin has its greatest value in the treatment of infections caused by gram positive bacteria. Nonetheless a great variety of gram negative as well as gram positive bacteria are killed *in vitro* by penicillin. As a rule greater concentrations are required for activity against the gram negative bacteria. Penicillin is bactericidal, and depending on environmental conditions and the nature of the bacterial species, lysis may accompany the death of exposed bacteria.

Staphylococci, pneumococci, and the aerobic spore-forming bacteria are the forms most readily lysed by penicillin. The rate of lysis seems to be dependent upon the normal rate of growth, occurring more rapidly at higher temperatures which would have the highest growth rates if penicillin were not present under the circumstances of cultivation.

The bactericidal action of penicillin seems to be expressed only upon



growing organisms. The metabolic activity of washed resting cell preparations seems not to be affected by penicillin. The addition of nutrient media to a resting cell preparation does result in a decrease in respiration. Under these circumstances the effects on respiration are apparently secondary to some effect on an anabolic process. Moreover, in the presence of a *bacteriostatic* agent penicillin no longer affects respiration indicating that the action of penicillin is on a growth process. As a matter of fact the bactericidal and bacteriolytic effects of penicillin can be abolished by the inclusion of

TABLE 64

*Demonstration of the ability of Staphylococcus aureus to undergo about one division in low concentrations of penicillin*

TIME	CONCENTRATION OF PENICILLIN					
	0.3 unit/ml			3 units/ml		
	Turbidity	Haemocytometer	Viable organisms	Turbidity	Haemocytometer count	Viable organisms
hrs		millions/ml			millions/ml	
0	0.28	180	—	0.28	180	200
1	0.33	200	214	0.31	168	81
2	0.43	176	161	0.38	160	61
3	0.40	180	70	0.42	160	21
4	0.30	80	34	0.36	126	8
5	0.23	86	32	0.33	116	0.4
6	0.18	24	0.06	0.30	80	0.7
7	0.14	—	0.1	0.20	18	0.8
24	—	1.4	0.001	0.13	1.2	0.004

The penicillin was added to the cultures immediately before incubation. The doubling (approx.) in turbidity in relation to the counts indicates the occurrence of some multiplication.

(From Chain and Duthie, 1945.)

unrelated bacteriostatic substances in culture media. The inability of penicillin to harm bacteria at temperatures below the minimum growth temperature is also in agreement with the findings suggesting the limitation of the activity of penicillin to periods of growth.

At a low concentration of penicillin bacteria may multiply for one or two divisions before a bactericidal action is noted. At higher concentrations a killing effect and lysis may be noted without a preliminary period of multiplication (Table 64). These data have been interpreted to mean that while penicillin may be limited to growing bacteria the action is not limited to specific events and metabolic reactions responsible for fission.

Bacteria are permeable to penicillin probably by the simple process of diffusion. The quantities of penicillin penetrating into bacteria may be

completely leached out by the mere process of washing except for organisms sensitive to penicillin which tend to retain about 750 molecules of penicillin per bacterium. Since the component binding the penicillin has not been isolated it is not possible to say whether the substance is actually a single or a number of substances and what metabolic roles are played in normal cell metabolism.

Strains of bacteria resistant to penicillin are derived from sensitive strains requiring amino acids as growth factors, and they can synthesize their own amino acids. On the other hand such resistant organisms do not differ from their sensitive parents in their ability to catabolize amino acids. Gale and associates have shown that penicillin interferes with the uptake of amino acids such as glutamic acid which must penetrate into gram positive bacteria by active transport. In general it can be shown that the concentrations of penicillin interfering with the active transport of amino acids are similar to those preventing growth. These and other findings argue in favor of a mode of action of penicillin which depends upon blocking the active transport of preformed amino acids required for assimilation into protein. Nevertheless three difficulties with this hypothesis have been pointed out:

- 1) While the inability of a bacterium to take up an amino acid required as a growth factor could account for inhibition of growth, it is not possible to visualize how this could explain the *rapid* bactericidal and bacteriolytic effects noted in many cases upon the addition of penicillin to growing cultures.

- 2) Gram negative bacteria not requiring amino acids as growth factors are sensitive to penicillin.

- 3) From a strain of *Bacillus subtilis* requiring amino acids and sensitive to penicillin a mutant has been derived which can grow on a medium with an ammonium salt as the sole source of nitrogen and yet which is inhibited by penicillin. To rescue the original hypothesis it might be proposed that penicillin can act in different ways upon different bacteria. Without further proof one might be reluctant to accept this hypothesis. However, in favor of this view, even if it does not prove the case, is the fact that in general, higher concentrations of penicillin are needed for bactericidal activity against gram negative bacteria that do not require amino acids than against sensitive gram positive bacteria.

While the difficulties mentioned do not make it clear as to how penicillin might act to interfere with the synthesis of protein, the notion of interference with protein synthesis still remains a valid concept. Most recently it has received important support from the finding by Hotchkiss (1950) that in suspensions of resting cells in the presence of penicillin there is an accu-

mulation of non-amino nitrogenous material which has the properties of peptides.

It has been shown that phosphate uptake continues in the presence of penicillin with concomitant increase in the amount of labile phosphorus per bacterium (Table 65) and that there is probably an accumulation of pentose complexes which contain labile phosphorus. Since such is the case penicillin may act by interfering with reactions in which these complexes participate. If the complexes act as coenzymes both in reactions of active transport and protein synthesis, it might be possible to draw into a unified picture all of the diverse data quoted. At the moment the lack of experimental studies makes this an idle speculation.

TABLE 65

*Phosphorus content of Staphylococcus aureus in the absence and in the presence of penicillin*

GROWING BACTERIA	BACTERIA AT ZERO TIME	65 MINUTES LATER	65 MINUTES LATER + PENICILLIN
Total phosphorus.....	14.2	13.9	22.4
Labile phosphorus.....	1.8	1.9	5.6

Phosphorus calculated as gamma per  $10^{10}$  organisms.

(From Park and Johnson, 1949.)

### *Resistance to Penicillin*

All bacterial strains sensitive to penicillin which have been studied are capable of giving rise to resistant strains. Many bacteria are also able to synthesize *penicillinase*, a specific enzyme inactivating penicillin. Depending on the particular strain, penicillinase occurs intracellularly, extracellularly, or both. The importance of penicillinase production in accounting for the resistance of mutants of sensitive strains is doubtful. It is suggested by some data (Table 66) that penicillinase production may be responsible for resistance to penicillin in some cases and not others. On the other hand there is no quantitative correlation between penicillinase production and degree of resistance. With a small inoculum penicillinase production does not seem to be effective in preventing the bactericidal activity of penicillin on sensitive bacteria while with a large inoculum of the same organisms the bactericidal action of penicillin is prevented.

A sensitive strain of *Staphylococcus aureus* has been trained to resist 60,000 times the quantity of penicillin to which it was originally resistant. This extraordinarily resistant organism showed a change in gram reaction



from positive to negative and an increased ability to synthesize the amino acids required for growth. A case of this character may well signify that the development of resistance to penicillin involves changes in metabolic patterns to types unaffected by penicillin, rather than the acquisition of mechanisms for inactivating penicillin.

While penicillin may kill the overwhelming number of bacteria in a sensitive culture, frequently the culture is not sterilized, and a variable number of survivors remain. Cultures derived from such organisms are known as *persisters* and are found to be sensitive to penicillin. Thus persisters are not insensitive mutants of the parent strain. The resistance of persisters to penicillin in the parent culture has been attributed to the possibility that they do not grow for reasons unknown and thus in spite of their sensitivity

TABLE 66

*Production of penicillinase by four different groups of staphylococci separated on the basis of their origin and sensitivity to penicillin*

ORIGIN AND NATURE OF STRAIN	NUMBER OF STRAINS	
	Total	Producing penicillinase
Naturally sensitive .....	8	0
Naturally resistant .....	2	2
Acquired resistance <i>in vitro</i> .....	3	0
Resistant strains recovered from patients treated with penicillin .....	9	7

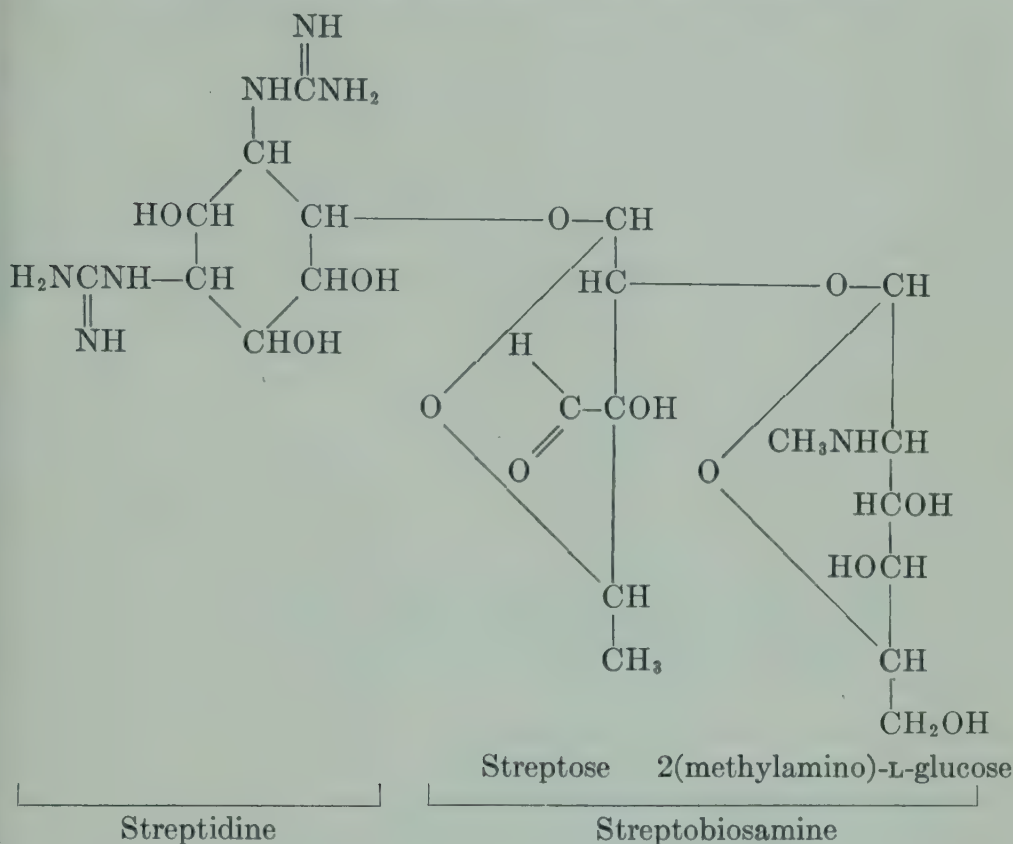
(From Blair, Carr and Buchman, 1946.)

do not succumb to the penicillin in their environment. In other words persisters are assumed to be in a state of dormancy. A definitive test of this hypothesis remains to be presented.

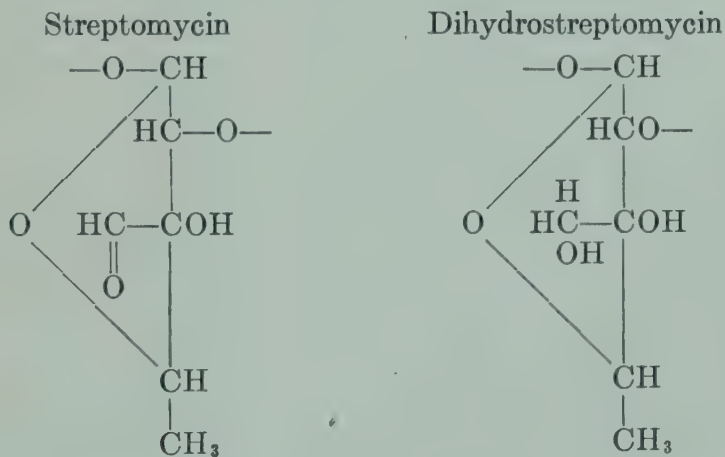
STREPTOMYCIN

The antibiotic streptomycin is a highly polar base produced by an actinomycete, *Streptomyces griseus*. It acts both as a bacteriostatic and as a bactericidal substance on a variety of species of *Eubacteriales* and actinomycetes. In the pH range from 5.2 to 7.7 the effectiveness of streptomycin increases about sixty-fold, the greatest activity occurring at about pH 7.8. This change is probably due to a greater capacity for the undissociated molecule to penetrate bacteria than the ion. Streptomycin has only a slight effect on fungi, for noticeable effects require of the order of ten thousand times the quantities potent for bacteria, and does not act at all on the protozoan *Entamoeba histolytica*.

The following structure has been determined for streptomycin:



Dihydrostreptomycin, because of its lower toxicity for animals, has tended to replace streptomycin in clinical applications. The structure of dihydrostreptomycin differs from streptomycin in that the carbonyl group of the streptose moiety is reduced:



A unit of streptomycin has been defined as the activity of one microgram of pure streptomycin base. For inhibition of the growth of acid fast bacteria *in vitro* the minimum amounts required are in the range of 0.1 to 1 microgram per ml. The conditions under which determinations of unit activity should be made have not been universally agreed upon by bacteriologists. For reproducible results it has been suggested that it is necessary to employ a standard inoculum of organisms, a standard time for reading growth in cultures, and that the medium employed should include known and constant amounts of inorganic salts and be free of substances adsorbing streptomycin. Preferably the temperature of incubation should be that optimal for growth of the assay organism.

The antibiotic activity of streptomycin seems to rest on a capacity to inhibit a respiratory mechanism. It is probably significant in this regard that the order of 500 times the quantities of streptomycin are required *in vitro* to inhibit the growth of clostridia as are needed for aerobic and facultative bacteria. Nevertheless, though relatively insensitive, clostridia are harmed by streptomycin, and in sufficient concentration streptomycin will agglutinate some bacteria and precipitate nucleic acid. These facts are indications that streptomycin can potentially be expected to act upon a number rather than a single cellular mechanism depending on the concentration. It has been argued that apart from anaerobes the primary action of streptomycin is on a respiratory process since respiration is affected by the same minimal concentration inhibiting growth.

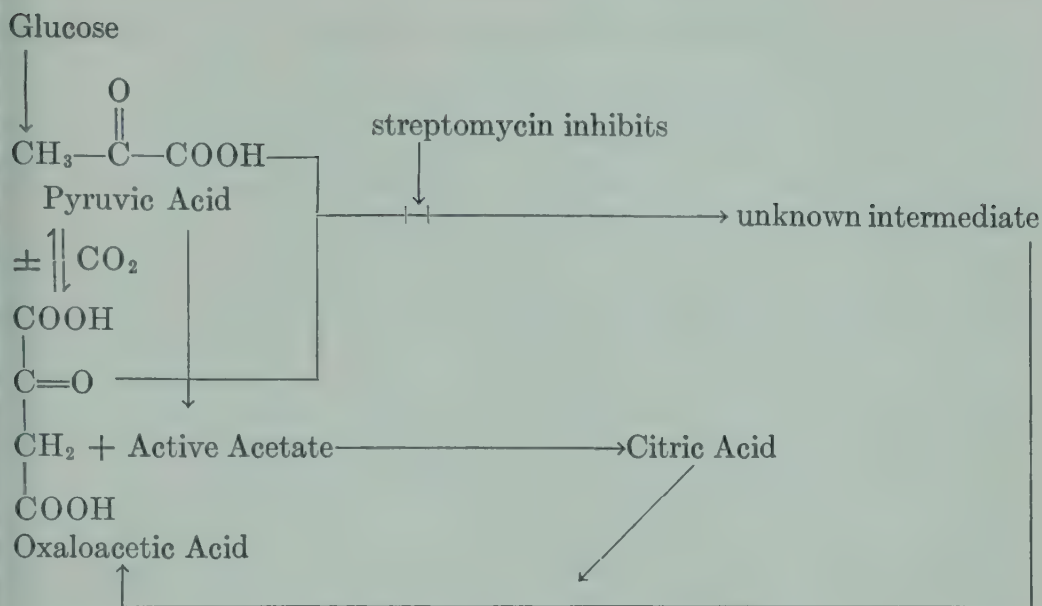
Unlike penicillin, streptomycin is adsorbed by both sensitive and insensitive species; thus the mere capacity of various cellular constituents such as nucleic acids to bind streptomycin is not a sufficient criterion for implicating such a reaction as a primary cause of interference with growth.

The interference of streptomycin with respiration has been postulated tentatively to rest on inhibition of the condensation of pyruvate with oxaloacetate to form intermediate metabolites. The scheme has been visualized by Umbreit and Oginsky, as shown on the following page. The claim that resistant bacteria do not exhibit the pyruvate-oxaloacetate reaction supports this scheme.

The comparative inability of streptomycin to inhibit the respiration of animal tissue in spite of the presence of the sensitive respiratory mechanism is probably due to the relative impermeability of animal cells to streptomycin. In addition there is some evidence indicating that the sensitive enzyme system is located within the interior of mitochondria. The inability of streptomycin to penetrate the mitochondria has been offered as a further explanation of the relative lack of sensitivity of animals to streptomycin.

A variety of bacteria sensitive to streptomycin including gram negative, gram positive, and acid fast species have been shown to be capable of yield-





ing mutants requiring streptomycin as a growth factor. What the metabolic role of streptomycin may be in this circumstance remains unrevealed. Any valid theory of streptomycin activity eventually must be able to account for this phenomenon.

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# Appendix

## TURBIDIMETRY AND THE ESTIMATION OF BACTERIAL POPULATIONS

When a beam of light passes through any substance, part of the light is scattered by the molecules of the substance. The nature of this phenomenon may be readily illustrated by recalling the visibility of a beam of sunlight entering a darkened room. Visibility of the beam exists because particles of dust, smoke, etc. deflect light rays toward the eye and away from the normal path of the beam. Conversely if light were not deflected a beam would be completely invisible except when looking straight down it into the light source.

At present the exact mechanism by which the light is scattered cannot be stated, since there are two differing theories for the process. Suffice it to say that light is scattered at all angles to a beam penetrating any material. In fact this statement is sufficiently general to include scattering by both particles large enough to be seen and the molecules of a gas. The general illumination of the sky is, indeed, an example of scattering of light by smoke, dust, water droplets, and molecules of gases. One would anticipate a darker sky in dry regions lying at high altitudes and away from cities. This observation is common knowledge, of course, and is even more pronounced at extreme heights of many miles where the daytime sky appears black except where the line of sight includes direct beams from the sun or stars.

In a similar way molecules of a pure liquid or a transparent solid scatter a portion of an incident beam of light. When a second material is dissolved in a liquid, the total intensity of the scattered light involves that from both solute and solvent. This relationship suggests the possibility of studying the intensity of light scattered by the solute by measuring that scattered from both the solution and the pure solvent and subtracting the second from the first. Experimentation early revealed the validity of this method and it is now widely employed.

Investigations of scattered light would have but little practical value unless the nature or the intensity of the scattered radiation bore some relationship to the scattering agent. It has been found that the more there is of a scattering agent and the larger the solute molecules (or particles), the greater the intensity of the scattering. Other important factors include the wave length of the light making up the beam, the intensity of the beam, and the difference between the refractive indexes of solvent and solute.

These various factors have been combined in an equation that permits



quantitative studies of light scattering. Important investigations have thus been conducted on solutions of proteins, synthetic high polymers, and other rather large molecules. Unfortunately for some problems, however, the present theory applies only to systems in which the particles are a good deal smaller than the wave length of the light used. Thus bacteria are too large for study by means of a quantitative theory applicable to molecules. Theoretical formulations have been sought for large particles but no really general results have been published.

However, on a somewhat empirical basis valuable use may be made of the phenomenon of scattering. An expression resembling Beer's Law is often obeyed over limited ranges of concentration. Therefore, as a necessary preliminary to an understanding of the equation for scattering, let us first explore the principles summarized in Beer's Law.

### *Beer's Law*

This relationship concerns the *absorption* of light by solutes and is expressed quantitatively as follows:

$$I = I_0 e^{-kcl} \quad (1)$$

where  $I$  is the intensity<sup>1</sup> of the beam after passing through the solution,  $I_0$  is the incident intensity,  $e$  is the natural base,  $k$  is a constant characteristic of the solute,  $c$  is the concentration of solute, and  $l$  is the length of the light path. Equation (1) essentially means that light diminishes in intensity as it passes through an absorbing material and that the extent of diminution depends upon the nature and concentration of the absorbing material and upon the length of the light path.

If equation (1) is rearranged and logarithms are taken

$$\log_{10} \frac{I_0}{I} = \frac{kcl}{2.303} \equiv acl \quad (2)$$

$a$  is variously called *absorptivity*, *absorbancy index*, or *extinction coefficient*. Obviously if this equation is obeyed any one factor may be evaluated when the others are known. Usually the equation is satisfied at least over certain ranges of concentration of a substance. Since it occasionally is not satisfied, a check must be made of each system using different concentrations. Once its applicability has been established, Beer's law can be widely used to de-

<sup>1</sup> In practice actual measurement is usually made of the total quantity of light involved rather than of the actual intensity. However, since the geometry of a given instrument is kept constant the data become light per unit where the unit may be difficult to evaluate but is constant nevertheless. As a result the term intensity is essentially a correct one.

termine the concentrations of many kinds of solutes. For this purpose the necessary measurements of light intensities are commonly made in colorimeters or spectrophotometers. These instruments record light intensities by means of photoelectric responses which may be made directly proportional to the quantity of light striking the light sensitive photoelectric cell.

Among the variables that must be well controlled is the wave length of the incident light since the extent of light absorption by materials varies with the wave length of the light. In addition compensation must be made for all intensity losses not due to absorption by the solute. Usually this adjustment is readily made by taking  $I_0$  as the intensity of the beam after it passes through the cell containing the solvent alone instead of the complete solution. In this way correction is made for reflections, scattering by the cell or solvent, and absorption by the solvent. Hence,  $I_0 - I$  represents only the light lost by absorption by the solute.

Nowadays many instruments are provided with simplified scales to facilitate use of the data. These ordinarily take a logarithmic form permitting the direct reading of  $\log_{10} I_0/I$  when the cell plus solvent is set equal to zero on the scale. The term  $\log_{10} I_0/I$  is variously known as the *absorbancy*, the *optical density*, or the *extinction*. Note that the extinction or optical density is a characteristic of both a particular substance and an instrument while extinction coefficient is a characteristic of the substance alone.

### *Turbidimetry*

This term is applied to methods for determining the concentrations of systems by means of the light scattered. There are, of course, many different applications, instruments, and methods, but we shall consider only those general principles that are readily applied to bacteriology. In general it will be convenient to assume that light losses by ordinary absorption do not occur, and most methods are designed to satisfy this assumption or to correct for any rather small absorption losses that do take place.

In discussions of light scattering the term turbidity is used to indicate the amount of light scattered during the passage of the beam. Clearly the turbidity is subject to measurement by two general methods. Light lost from the beam is equal to the light scattered (when absorption is absent or eliminated) or the light actually scattered may be measured directly. This latter procedure is occasionally called *nephelometry*.

Since light is scattered in all directions and since the extent of scattering varies with the direction, it becomes difficult to determine by direct measurement the total amount of light scattered. This problem may be avoided, however, for the quantity of light scattered in a given solid angle by a particular material is directly proportional at low concentrations to the number of particles. The intensity of the scattered light is very low, and

extremely sensitive instruments are required for its measurement. Although little use has so far been made of them in bacteriology, instruments are now available which record the quantity of light scattered by solutions in one or more directions in fixed solid angles. Potentially the methods involved will determine relatively small concentrations of bacteria ( $10^4$  *Escherichia coli*/ml.) in special media or when the organisms are resuspended in solutions free of colloidal particles. The relationship required is

$$I_s/I_0 = Kc \quad (3)$$

where  $I_s$  is the quantity of light scattered in the given solid angle,  $I_0$  is the quantity of light in the incident beam,  $c$  is the concentration, and  $K$  is a constant whose units depend upon those of  $c$ . Clearly the value of  $K$  must be predetermined for each system by means of known values of  $c$  which must in turn be obtained by some independent method. Concentrated suspensions may be diluted to a suitable range for operations with a particular instrument.

Whereas the preceding scheme may be applied to advantage when fairly dilute suspensions are available for analysis and further dilution is undesirable, such limitations are usually not imposed. More often an investigator desires knowledge of the number of bacteria in a more densely populated culture. In this event a spectrophotometer or even a colorimeter will provide turbidity data in the following way.

An equation that relates the concentration of particles to the absorbancy (optical density) may be written as

$$\log_{10} \left( \frac{I_0}{I} \right) = \tau lc \quad (4)$$

The various letters have the same meanings as in equation (1) except that  $\tau$  is a constant called the *turbidity coefficient*. For bacteriological purposes  $c$  is expressed as organisms per ml. and  $l$  may be incorporated into  $\tau$  providing test tubes or other appropriate containers of a given size are always used.  $\log_{10} \left( \frac{I_0}{I} \right)$  is determined just as was described in the discussion of Beer's law. As in the cases of the direct measurement of scattered light and in the determination of concentration by light absorption, the method must be calibrated to provide data on a constant (in this case  $\tau$ ).

For this purpose  $I_0$  is evaluated using a tube of ordinary medium. With cells in the same medium  $I$  is then read in a matched tube. Readings are repeated at various dilutions of cells, and the exact concentrations are determined by an independent method, the most useful being dilution followed by plate count or microscopic count in a Petroff-Hausser chamber. Once this has been done it is customary to plot  $\log_{10} \left( \frac{I_0}{I} \right)$  against number



per ml., and unknown concentrations are read later from this calibration curve.

Several important points of operation should be borne in mind although the difficulties represented can usually be circumvented. In the first place the quantity of light scattered depends upon the wave length, hence any change in this factor requires recalibration. In this connection it should be pointed out that the scattering increases very greatly as the wave length decreases. Stated in other words, the method will be more sensitive the shorter the wave length until light absorption becomes significant. It will be recalled that we originally assumed lack of appreciable light absorption by the solute. Although failure to satisfy this condition may be overcome by means of special calibration studies, they will ordinarily be complex and vary from case to case.

Turning now to light absorption by the medium, one may still work with equation (4) providing that this absorption is not altered by the growth of the organisms. If it is altered the wave length may be varied or the bacteria removed, washed, and resuspended in fresh solution. Likewise, the scattering of the medium (or suspending solution) should be constant and reasonably low when compared to the scattering of the bacterial suspension.

A third point of operation concerns the calibration curve. At low concentrations the curve will probably be linear or nearly so, but at higher cell counts a considerable departure from a straight line will occur. At still higher points increased concentration will not increase  $\log_{10} \left( \frac{I_0}{I_2} \right)$  at all, hence, high cell populations cannot be determined precisely unless they are first diluted to a more suitable range. The location of the range desirable for turbidimetric determination must be found by experiment for each system.

Finally changes in the organisms themselves will affect the determination of their number. It is known that the scattering of light depends upon the shapes of large particles as well as on their sizes. Therefore, if the population is not statistically constant in size, cell shape, and chain length the turbidity measurements will be altered. As has been pointed out elsewhere, such changes normally do occur in bacterial cultures with aging, the presence of harmful agents, and similar factors. Furthermore, when cells lyse for any reason the resulting debris scatters light although it does not represent viable cells and does not even compare quantitatively in effect with intact cells, alive or dead.

An illustration of a change in nephelometric reading without a change in the number of bacteria is provided by experience in measurement of the turbidity of bacterial suspensions subjected to supersonic vibration. Immediately after the application of the sonic energy is begun there may be

an increase in readings of turbidity. This increase is due to the fact that any clumps of bacteria in the suspension are dispersed into the individual organisms as the first effect of the sonic energy. Thus though there is no change in the total amount of material (bacteria) contributing to light scattering, a decrease in the average particle size results in an increase in scattering in this particular size range.

The preceding precautionary discussion is not intended to discourage use of the method but merely to call attention to potential errors and thereby aid in avoiding them. As a matter of fact turbidity measurements are now routine in bacteriology and biochemistry since they save a great deal of arduous and lengthy counting.

Commonly such methods are calibrated readily by direct microscopic counts permitting adjustments for cell pairs and the short chains which sometimes occur. If data of this type for *Escherichia coli* are compared with plate counts, the latter are found to be about twenty per cent lower. This discrepancy may arise from counting of nonviable cells in the Petroff-Hausser chamber and the counting of all pairs or chains as single organisms in the plating technique. Since the methods of calibration can differ somewhat, this difference must be considered in systems where it may become important.





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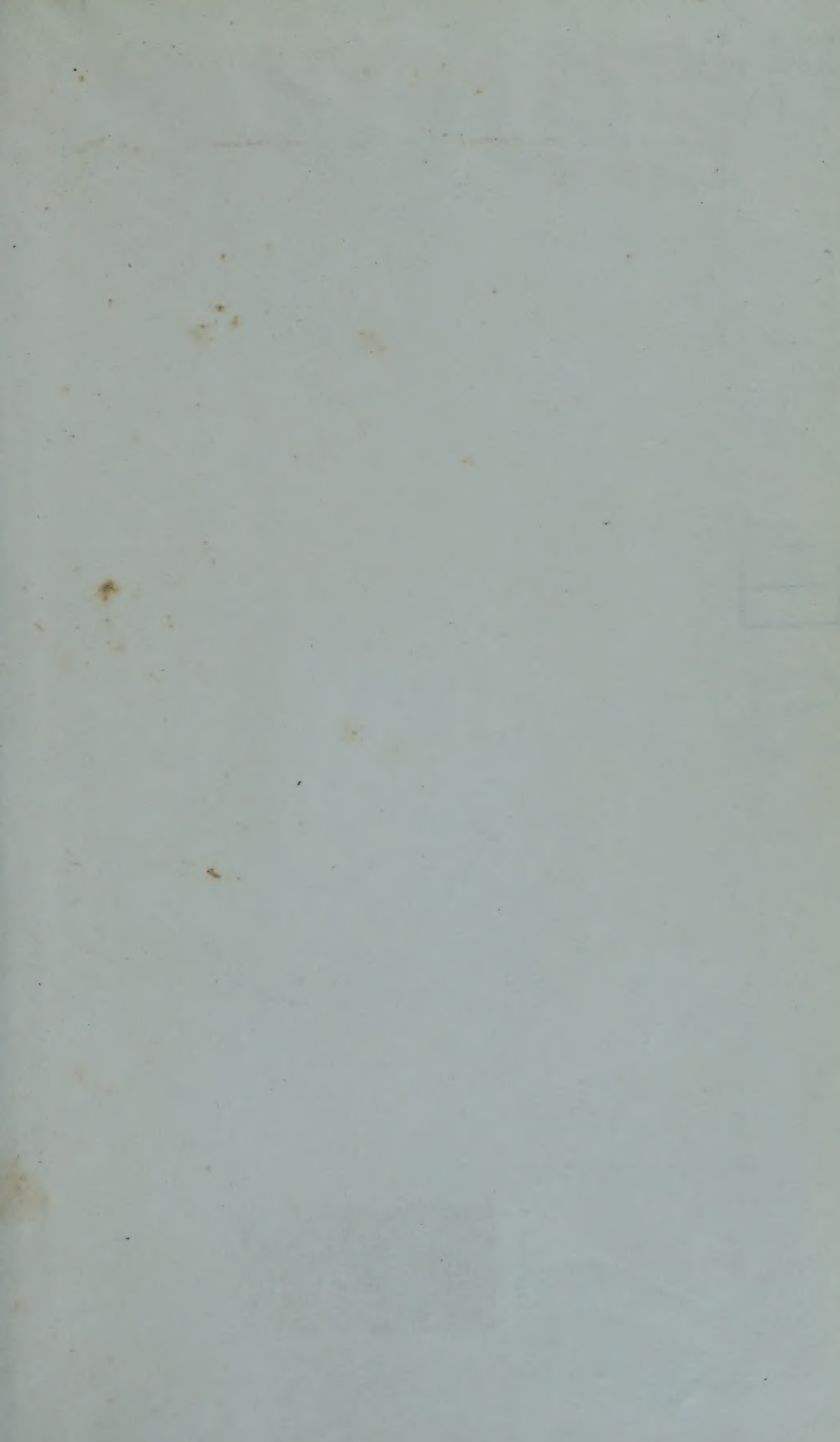
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